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ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

Development of White Mould-Resistant Soybean with Oxalate Oxidase and through the Identification of Resistance Genes

REPORT No. OREP - 1999/02

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FORWARD

The Ontario Research Enhancement Program (OREP) is a $4-million two-year federal research initiative that is administered by the Research Branch of Agriculture and Agri-Food Canada (AAFC), with input from the agriculture and agri-food sector, universities and the province. Research focuses on two areas identified by the sector as:

1. Priorities responding to consumer demand for higher quality products; and
2. Ensuring crop-production management systems are environmentally sustainable.

Biotechnology is one of the areas to be explored. Ontario's agricultural production is based primarily on growing diversified crops using intensive production practices, but long-term viability is linked to the development of sustainable crop production management systems.

There are also opportunities to enhance the economic contribution of Ontario's agriculture and
agri-food sector by adding value to the diversified primary commodities produced in the province. The Program is expected to be of particular interest to the corn, soybean, greenhouse, fruit and vegetable sectors and the emphasis will be on projects focussing on food quality improvement and sustainable crop production management such as:

1. The development of value-added animal and crop food products and ingredients that meet domestic and export market demands, with a particular emphasis on quality issues such as processing, packaging, shelf life, new product and ingredient development, high-value alternative uses, probiotics and functional foods;
2. Biotechnologies to reduce environmental pressures related to crop protection practices; and,
3. Integrated crop protection and production practices for both field and greenhouse crops.

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EXECUTIVE SUMMARY

White mould, caused by the fungal pathogen *Sclerotinia sclerotiorum*, is a major and economically devastating disease of soybeans in Ontario; it reduces yield and results in revenue losses of about $6 million annually. Seed contaminated with fungal sclerotial bodies compromise soybean quality and is rejected by importers of food grade soybeans. The goal of this project was to develop white mould-resistant soybean, thereby increasing yield, reducing environmental pressure and enhancing our export opportunities.

There are no commercial soybean varieties with high levels of resistance to white mould. Two complementary strategies to develop white mould-resistant soybean were explored:

1. Transformation of soybean with the wheat germin gene (*OxO*) which encodes for oxalate oxidase (OxO), an enzyme that targets oxalic acid secreting pathogens.
2. Identification of molecular markers linked to resistance genes for development of white mould tolerant/resistant soybean germplasm.

This project has shown rapid progress toward achieving white mould-resistant soybean. The major goals of the four objectives have been met as follows:

1. **Localization and quantification of oxalic acid in *Sclerotinia sclerotiorum* -infected soybeans.** Oxalic acid accumulation was examined in growing tip, leaf, or leaf node/stem tissue and petals. Accumulation was highest in leaf, growing tip and petals indicating that this tissue was most susceptible to white mould infection. Most significant, oxalate started to accumulate on all tissues between 24 and 48 hours and peaked by 72 hours. This information is very important for studies on plant defense mechanisms.

2. **Development of suitable screening methods for white mould resistance.** Growth room screening identified the 'seedling cut stem' inoculations, coupled with a newly developed inoculation medium, to be the most reliable. It distinguished differences between S19-90 (resistant) and Elgin 87 (susceptible) varieties, and between transgenic lines and those of parental and null lines. Natural disease development in white mould nurseries was very poor, hence preventing field evaluation of resistance. However, field and growth room inoculations identified several resistant plant introductions that were subsequently incorporated into soybean breeding programmes at GPCRC and ECORC. The new screening techniques should permit effective screening of progeny and development of commercial varieties.

3. **Transformation with the wheat germin *OxO* for resistance to white mould.** Several transgenic lines have been identified as highly resistant to white mould. The transgene product, oxalate oxidase, was found to be identical in structure to the wheat germin homohexamer, the source of the gene. The most advanced transgenic line, 80-30-1 has undergone two years of confined field trials at a total of 7 different sites in Ontario and Quebec and has shown superior resistance to white mould without any loss in yield. This line shows a variable level of oxalate oxidase activity which may explain its incomplete resistance. Other transgenic lines with consistently higher levels of resistance are in the pipeline.

4. **Development of molecular markers linked to genes providing resistance to *S. sclerotiorum*.** Five plant introductions with resistance superior to the best commercial line S19-90 have been identified and crossed into susceptible lines to generate genetic mapping populations of Recombinant Inbred Lines (RILs). Unfortunately, hot weather conditions prevented white mould development and precluded evaluation of resistance of the RILs. Simple Sequence Repeat (SSR) DNA molecular markers were used to profile resistant and susceptible germplasm, including the parents of the RIL populations. Analysis of the SSR allele patterns identified four candidate genomic regions which can be rapidly tested for the presence of resistance loci once the populations are ready. This will give us the molecular marker technology to identify breeding lines with white mould resistance genes and accelerate development of resistant lines.

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INTRODUCTION

White mould, caused by the fungal pathogen *Sclerotinia sclerotiorum*, is a major and economically devastating disease of soybeans in Ontario, northern USA and other parts of the world including Argentina and China. In Ontario, yield is reduced by an average of 8% for each 10% increase of plant infection resulting in revenue losses of about $6 million annually. Management practices, such as narrow row spacing which increase yield, also increase the incidence of white mould. Seed contaminated with sclerotial bodies compromise soybean quality and is rejected by many importers of food grade soybeans. No source of complete white mould resistance has been identified.

Molecular markers and associated technologies can be very efficient in the analysis of the genetic basis of quantitative traits and marker-assisted selection (MAS) for disease resistance breeding (Staub et al., 1996). Use of molecular markers to identify quantitative trait loci (QTL) has proven very efficient in soybean breeding programs (Concibido et al. 1996, Mansur et al. 1996). Programs in Ontario, Michigan, and Ohio have initiated projects on MAS for white mould resistance/tolerance. USDA has identified restriction fragment length polymorphism (RFLP) probes which are polymorphic on elite US germplasm. Microsatellite DNA markers have been shown to be a very powerful tool for characterizing soybean genotypes and for MAS (Cregan et al., 1994, Rongwen, et al., 1995, Powell et al., 1996). In soybeans, Simple Sequence Repeat (SSR) molecular markers have higher levels of heterozygosity than the other molecular markers (Powell, et al., 1996), are generally applicable to the species, rather than a specific cross, and are not encumbered by proprietary patents (Staub, et al., 1996).

To date, progress in the development of selectable molecular markers suitable for marker-assisted breeding for white mould resistance is limited but encouraging. It has been reported that Asgrow A2506 and Novartis S19-90 have partial resistance in the field and Asgrow WR2347 and Williams 82 are susceptible (Kim et al 1999). F2, F3 and F2 derived F3 families derived by inter-mating the first three of the above lines and evaluated in the greenhouse indicated that multiple genes, some of which may be recessive, condition this partial resistance (Hoffman et al 1999). The chromosomal location of these genes is still unknown. However, resistance gene analogs have been isolated and mapped in soybeans (Kanazin et al 1996; Yu et al 1996). They cluster with each other and with known disease resistance genes and these clusters are widely distributed throughout the genome. Such clusters may well be associated with the genes conferring resistance to white mold.

In the field, white mould disease symptoms are generally not observed until late flowering, and do not become pronounced until the seed pods begin to develop (Athow 1987). Infection is most often initiated at stem nodes by airborne ascospores that germinate on and colonize senescing flowers or leaves (Martens et al. 1988). The infection then moves rapidly through the stem. The role of oxalic acid in pathogenicity of *S. sclerotiorum* is well documented (Dutton and Evans 1996). Oxalic acid minus mutants of *S. sclerotiorum* are not pathogenic (Godoy et al. 1990). Maxwell and Lumsden (1970) reported that within 2-4 days following inoculation of bean hypocotyls with virulent *S. sclerotiorum* isolates, oxalic acid accumulated 20-40X in the hypocotyls, and that this accumulation correlated with an increase in disease symptoms. Although oxalic acid has been identified as a critical factor in *S. sclerotiorum* pathogenesis, it is not clear at what stage oxalic acid accumulates during the natural course of the disease, or in what host tissue(s).

Oxalic acid produced by the fungus during the infection process weakens plant cell walls by chelating the cell wall calcium and by activating cell wall digesting enzymes at the low pH. This action provides the fungus with nutrients. Therefore it follows that pathogen invasion would be inhibited by enzymes capable of degrading oxalic acid. Lane et al. (1986) first proposed that wheat germin may have an oxidative role in plant defence. The wheat germin gene (Lane 1991) encodes the enzyme oxalate oxidase (OxO) which catalyses the oxidation of oxalic acid to hydrogen peroxide (H_2O_2) and carbon dioxide as shown in the following chemical reaction:

$$\underset{\text{oxalic acid}}{[COOH]_2} + 2O_2 \xrightarrow{\text{OxO}} H_2O_2 + 2CO_2$$

It would be expected that wheat germin OxO would block oxalate accumulation and thereby *S. sclerotiorum* invasion. In addition, H_2O_2, a product of this reaction, appears to have important functions in activating the initial stages of the plant defence response (Baker and Orlandi 1995, Lamb and Dixon 1997) including:

1. cell wall cross-linking that renders the cell walls refractory to digestive enzymes (Bradley et al. 1992);
2. triggering programmed cell death (hypersensitive response) which inhibits pathogen invasion (Keen 1992, Brisson et al. 1994, Levine et al. 1994);
3. inducing plant defence genes (Levine et al.1994);
4. mediating biosynthesis of antimicrobial proteins (e.g. phytoalexins, lytic enzymes) (Lamb et al. 1989); and,
5. indirectly mediating systemic acquired resistance through the hypersensitive response (Klement 1982, Ryals et al. 1994).

These attributes indicate that the wheat germin gene was an excellent candidate for soybean transformation to provide resistance to oxalic acid secreting pathogens.

We have used two complementary strategies to develop white mould-resistant soybean:

1. Transformation of soybean with the wheat germin gene (*OxO*) that encodes a homohexameric oxalate oxidase (OxO), an enzyme that catalyses the oxidation of oxalic acid; and,
2. Identification of molecular markers linked to resistance genes for development of white mould tolerant or resistant soybean germplasm.

Both of these strategies require a reliable method(s) of white mould resistance screening to identify tolerant/resistant germplasm and assess resistance in transgenic plants. Numerous techniques reported include, exposure to oxalic acid (Tu 1985, Rowe 1993), inoculation of severed stems (Chun and Lockwood, 1984) and nodes (Cline and Jacobsen, 1983) and growth room evaluations (Boland and Hall, 1986). Recently, 15 commercial soybean cultivars were evaluated in the field and in the laboratory using the excised stem technique. In the laboratory, Maple Presto, McCall and Clay were most resistant and in the field, Portage, Maple Presto and McCall were most resistant. However, there was no correlation between laboratory and field evaluations when all entries were considered. The authors concluded that the laboratory test measuring stem decay would not be a reliable method to screen for a breeding program (Nelson et al. 1991). Others have had more success. In a comprehensive study in Iowa, the responses of 12 soybean cultivars to *S. sclerotiorum* were evaluated in a controlled environment and in the field.

Several methods were used to screen plants in the controlled environment including lesion size on detached leaves, stems inoculated with mycelial discs, incidence of stem rot following foliar inoculation, lesion lengths on stems following exposure to oxalic acid and levels of soluble pigments in treated stems. Results varied with method and cultivar but Corsoy 79 and S19-90 were most resistant by all methods. Oxalic acid treatment and a detached leaf assay were better than mycelial inoculation of stems and foliage to evaluate field resistance (Wegulo et al. 1998). Light intensity has been found to be very critical to soybean cultivar response to *S. sclerotiorum*. Cultivars can be grouped based on photon flux density of photosynthetically active radiation (PAR). PAR sensitive cultivars become more resistant with increased PAR and the response of PAR insensitive cultivars remains unchanged at different PAR intensities. Light sensitivity may complicate laboratory/growth room screening attempts to correlate with field tolerance (Pennypacker and Risius, 1999).

All of these methods were successful in distinguishing differences among varieties but there was very little field data to confirm results of the laboratory tests. After several years of observations, certain commercial varieties have been recognized as being tolerant to white mould. These varieties and recently identified plant introductions have provided material to improve screening techniques and identify the one(s) in which laboratory and field data correlate.

OBJECTIVES

Oxalic acid is identified as a critical component in *S. Sclerotiorum* pathogenesis yet it is not clear at what stage oxalic acid accumulates during the natural course of the disease, or in what host tissue(s). As this information is important to the transformation studies, the first objective was:

1. **Localization and quantification of oxalic acid in *Sclerotinia sclerotiorum*-infected soybeans**
   The development of white mould-resistant soybean relies on effective screening for white mould resistance that can be applied in the growth room and under field conditions. Therefore, the second objective was:

2. **Development of suitable screening methods for white mould resistance**
   Two complementary strategies have been used to develop white mould-resistant soybean:

3. **Transformation of soybean with the wheat germin *OxO* for enhancing disease resistance**

4. **Development of molecular markers linked to soybean genes that provide resistance to *Sclerotinia sclerotiorum***

MATERIALS AND METHODS

1. Localization and quantification of oxalic acid in *Sclerotinia sclerotiorum* -infected soybeans

1.1 Fungal isolates and growth conditions.

Four *Sclerotinia sclerotiorum* isolates (obtained from Dr. T. Anderson; AAFC, Harrow), from mung bean, soybean and hemp were used in preliminary studies of acid production. Soybean isolate Soy2 was used for all other experiments. Cultures were routinely grown at 24°C in darkness on potato dextrose agar (PDA) medium. For long-term storage, sclerotia were harvested from plate cultures, dried for two weeks at ambient temperature in a desiccator chamber, and then stored at -20°C (Huang et al. 1998). Cultures were reactivated by placing individual sclerotia onto PDA.

For growth experiments, 35 mL liquid PD medium was inoculated with four plugs (approx. 4-mm diam.) of mycelia from seven-day-old cultures grown on PDA, and incubated at 24°C in the dark, without aeration. To measure fungal biomass, cultures were harvested by filtration through pre-weighed Whatman #1 filter paper, and the sclerotia were separated from the mycelia. All fungal tissue was dried at 60 C for 24 h, then placed in a desiccator chamber for 24 h before dry weights were measured.

1.2 Infection assays.

Soybean (cv. Harosoy) seeds were sown in 10 cm diam. pots (four seeds per pot), and grown for two weeks in a growth room under fluorescent and incandescent lighting (16-h photoperiod/25°C daytime temperature, 20°C night-time temperature). To prepare the inoculum, a desiccated sclerotium of the soybean isolate Soy2 was placed in the centre of a 90-mm petri dish containing PD medium (Difco Laboratories) amended with 0.5% agar, and incubated in the dark at 24°C for 5 days. When the mycelia had covered approximately 2/3 of the plate, strips of mycelia (approx. 1 cm wide x 3-4 cm long) were scraped from the growing edge of the colonies, taking care to include as little agar as possible. The scrapings were placed into a sterile syringe and forced through a 26-g needle into a second sterile syringe. This procedure was repeated five to six times to produce a homogeneous inoculum. Droplets of inoculum (30 uL) were applied to the plants using a micropipettor, and pipette tips that had been cut at the narrow end to produce an approximately 1-mm diam. opening. Plants were inoculated 1) between the three leaflets of the growing tip, 2) on a primary leaf, to one side of the central vein, or 3) at a primary leaf node. Control plants were inoculated with homogenized PDA. Due to the viscosity of the inoculum, the pipette tips were changed frequently during the process. The plants were covered with clear polyethylene bags (# 11) and returned to the growth room. After 48 h, the bags were removed.

1.3 Oxalic Acid Assays.

Acid production was assessed qualitatively by growing the *Sclerotinia* isolates on a medium with the pH indicator bromophenol blue, as described by (Godoy et al., 1990). For quantitative assays, culture supernatants or plant extracts were used. The culture supernatants were separated from mycelia of *S. sclerotiorum* cultures by filtration through Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) and assayed directly. Infected (visible lesions) or mock-inoculated plant tissue was excised, weighed and pulverized (2 x 45 sec, speed setting #4) in 500 µL 0.5 N HCl, using the FastPrep FP120 Instrument (Bio 101, Inc., Vista, CA), and the FastDNA® ¼" sphere + garnet Lysing Matrix Combination (Bio 101, Inc.). Samples were centrifuged for 10 min at 13000 rpm in a microfuge, and 400 µL supernatant removed for assay. Extracts that were not assayed immediately were stored at -20°C.

Oxalic acid levels in culture filtrates and plant extracts were routinely determined using a commercial colorimetric-enzymatic assay (Sigma-Aldrich Canada, Ltd., Oakville, ON). Prior to assay, extracts and culture filtrates were processed according to the recommendations provided with the assay protocol. Briefly, an equal volume of 10 mM EDTA was added to each sample, and the pH was adjusted to between 5 and 7. Approximately 45 mg activated charcoal was added, and the samples were incubated at room temperature for 5 min with gentle mixing. Samples were centrifuged at maximum speed at room temperature in a microfuge, and 0.8-1.0 mL of the supernatant was retained for assay. The assay was carried out according to the assay kit protocol, except that the reactions were scaled down five-fold for use in a microtitre plate format, and the absorbance was read at 595 nm, using a microplate reader (BioRad model 550). These modifications did not affect the linearity and reproducibility of the assay for oxalate concentrations between 0.1 and 1 mM (K.F. Dobinson, K. Hawley and M. Cowan, unpublished).

2. Development of suitable screening methods for white mould resistance

2.1 Evaluation of commercial varieties and plant introductions for resistance in the field.

A 1-ha field screening nursery was established at GPCRC by spreading 1 tonne of sclerotia obtained from a commercial white bean cleaning process. The sclerotia were broadcast in the fall and incorporated just below the surface using drag harrows. The selected field consisted of a loam soil with high organic matter, pH 6.7, protected from the prevailing winds by a low ridge and tree line. Provision was made for overhead irrigation. Varieties and plant introductions (PIs) were planted in 5-m rows, 50 seed/row with a row spacing of 60 cm. Each entry was replicated 3 or 4 times depending on the trial and seed availability. Trials were planted the last week of May or the

first week of June depending on soil conditions. Observations of percentage plants infected or disease severity were made in August and the first two weeks of September. Final counts and observations were made in mid-September prior to total leaf fall. Trials were overhead irrigated for 1 h daily from July 15 until mid-September each year to ensure adequate moisture and humidity for disease development and spread. Controls were included in all trials. Elgin 87 (susceptible control) was included in all trials, every fourth row to provide a comparison for infection. Williams (susceptible) and S19-90 (resistant) were also included in most trials for additional controls.

In 1998 and 1999, natural infection in the nursery failed to develop, in spite of frequent irrigation and suitable environment, apothecia did not develop. In 1999, resistance of commercial varieties were evaluated by inoculating cut stems (5/row) on 11/08/99. Stems were cut approximately 60 cm above the soil surface and inoculum was applied with a syringe as in growth room studies (see below). An additional 5 plants/row were also inoculated by inserting infested oat kernels into wounded stems. Infection developed in all plants inoculated by the "cut stem" method. Infection was inconsistent with oat kernel inoculation. Length of living stem was measured from the soil line to the bottom of the disease lesion on the cut stem-inoculated plants at leaf senescence.

2.2 Growth room evaluation of inoculated cotyledons and cut stems of seedlings as indicators of resistance.

Several standard culture media were screened for growth and suitability as inoculum for plants in the greenhouse and growth room. These were modified further to promote adhesion to plant parts under humid conditions and to promote growth of the pathogen. Wheat starch was added to all media at 0, 5, 10, 20 and 40 g/l. The final three media were as follows: 1) ½PDA-S [potato dextrose agar (Difco) at 10 g/l H_20 (½ normal strength) with wheat starch]; 2) DA-S [dextrose 10 g, agar (Difco) 20 g/l H_20 and wheat starch]; and 3) A-S agar 20 g/l and wheat starch. Growth rate on the media was determined by inoculating the centre of each plate with a 0.5 cm disc of inoculum from an actively growing colony of *S. sclerotiorum* and measuring colony diameter at 24, 48 and 72 h. Sclerotia were harvested, dryed and weighed after 4 d growth in the dark at 24°C.

Soybean varieties and PIs were evaluated for resistance in the growth room to confirm field observations and to determine if varieties could be screened at the seedling stage. Seedlings were grown in 4-cm fibre pots or 2.5-cm transplant cells in ½ sand, ½ peat medium at 26°C with 14 h light. Seedlings were inoculated 1-2 weeks after planting. Cotyledons were wounded with a needle and inoculum was placed on the wound (1 cotyledon/plant) or plants were cut below the primary leaves and inoculum was placed on the cut stem. The inoculum consisted of homogenized

3-day old culture grown on ½ PDA-S + pectin (to each plate of 15 ml ½ PDA-S was added 0.5 g of pectin) and was applied with a syringe equipped with a large bore needle (#15GP). Following inoculation, plants were placed in a humidity chamber for 3 days. Plants were subsequently placed in a non-humid location for 3-7 days prior to rating resistance by counting dead plants or measuring the resulting lesion.

2.3 Screening transgenic material for resistance

Transgenic soybean lines obtained from ECORC were evaluated in the growth room and field for resistance to white mould. Methods for inoculating the cotyledons and cut stems of seedlings were similar to those used for commercial lines and plant introductions in the growth room (see above).

One field trial was conducted in 1998 with transgenic material. Soybean lines were planted in single 5-m rows, 50 seed/row with a row spacing of 50 cm, replicated 8 X. Stems were detached below the first trifoliolate leaf after it had expanded completely and the cut surface was inoculated. The first inoculations were completed on 23/07/98 and rated on 07/08/98 and 01/09/98. The second inoculations were made on 20/08/98 and rated on 01/09/98 for lesion development. Inoculated stems were covered with plastic drinking straws for 2 d to prevent dessication and permit infection to develop (5 plants/row). A second inoculation method involved inoculating an unwounded plant node with ½PDA-S (5 plants/row). Plots were subjected to overhead irrigation 1 h/d until rated for disease severity.

3. Transformation with the wheat germin *OxO* for resistance to white mould

3.1 Plasmid constructs

The following plasmids contain the wheat germin gene that was originally characterized by one of us, B. Lane. The wheat germin gene, a 700-bp fragment coding for OxO and a transit peptide was derived from the *gf2.8* fragment of pGermin I (Lane et al. 1991).

1. *pFF19germin:* the pFF19 cassette has the CaMV 35S enhancer, promoter and polyadenylation signal (ΦA), and a multiple cloning region between the promoter and the termination sequences in pUC120 (Timmermans et al. 1990). The 700-bp fragment was ligated into the multiple cloning site.
2. *pRD400germin:* constructed from pFF19germin and the binary vector pRD400 (Datla et al. 1992). pRD 400 is identical to the commonly used binary vector pBin19 (Bevan 1984) with the exception of an alteration in the coding region of the kanamycin resistance gene, *nptII*. The

CaMV 35S enhancer-promoter-germin-ΦA35S (*HindIII/EcoR1*) fragment was ligated to the linearized pRD400 that had been digested with *HindIII* and *EcoR1*.

3. *pHyg^R:* contains a coding region for hygromycin phosphotransferase (*hph*) that is regulated by the CaMV 35S promoter and the 3' nos terminator in pUC119.
4. pFF19sox ΦAnat: the germin gene is regulated by the 'superpromoter' that is comprised of three copies of the *Agrobacterium* octopine synthase (*ocs*) enhancer, the mannopine synthase (*mas*) promoter (Ni et al. 1995) and a polyadenylation sequence from the native germin gene (ΦAnat)); the CaMV 35S enhancer and promoter have been deleted from the pFF19 cassette.
5. pTZ19RtCUPΔOx ΦAnat: the germin gene is regulated by the tCUPΔ promoter and the CaMV 35S 3' nos terminator in pTZ19R (Pharmacia; modification of pUC119). The tCUPΔ is modified tCUP (Foster et al. 1999). The NdeI site at position #2084 of the promoter (30 bp upstream of the translation initiation) was changed to BglII by PCR. An NcoI site was added at the initiator methionine codon, and the tCUP 30 amino acid and GUS fusion peptide was deleted. The 2.2Kb tCUPΔ plus 2.8Kb pTZ19R plasmid fragment was ligated to the Ox NcoI site which had been introduced at the coding start site.

3.2 Transformation, selection and plant regeneration.

3.2.1 Agrobacterium-mediated transformation:

Transformation of wounded cotyledonary explants of soybean breeding line X2653-4-3-S2-2 was similar to that described previously (Donaldson and Simmonds 2000). Wounded explants were co-cultured with *A. tumefaciens EHA105* carrying the binary vector *pRD400germin.* Shoots recovered on kanamycin selection (300 mg/L, Sigma) were assayed for OxO activity (see below) to confirm transformation. Transgenic shoots were transferred to rooting media [half-strength B5 medium (Gamborg 1982) with 1% sucrose, 0.2 mg/L NAA and 0.6% Phytagar (Sigma)], and when rooted were potted in synthetic soil (Sunshine mix #3, Sun Gro Horticulture Canada Ltd.)). At the two trifoliolate leaf stage the plantlets were transferred to soil (3:1:1:1 mix of ASB Original Grower Mix (a peat-based mix from ASB Greenworld, Ontario):soil:sand:perlite) and grown to maturity in growth cabinets at 25°C under a 16-h photoperiod with a light intensity of 360-385 $\mu molm^2s^{-1}$ provided by cool white fluorescent and incandescent lamps.

3.2.2 Biolistics-mediated transformation:

Gold particles, 1 μm in diameter, were coated with DNA consisting of a mixture of two plasmids, one carrying the selectable marker gene *Hyg^R* and the other carrying a plasmid incorporating the wheat germin gene *OxO*. The gold carriers were bombarded into the target tissue by means of the Biolistic Particle Delivery System (Bio-Rad) as described previously (Simmonds and Donaldson 2000). The target tissues were embryogenic proliferative liquid cultures of short-season soybean breeding lines X2650-7-3-2 or OT-95-10, a small seeded natto type and a large oilseed line,

respectively. Initiation and maintenance of embryogenic cultures, selection of transgenic clones, maturation of transgenic embryos and regeneration of transgenic plants was similar to that previously described (Simmonds and Donaldson). Transgenic plants were identified by means of the OxO qualitative activity assay (see below).

3.3 Analysis of putative transgenic plants.

3.3.1 Southern analysis:

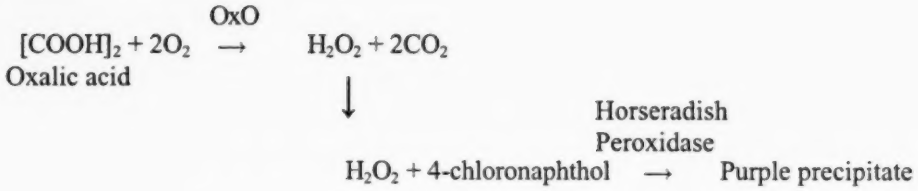
Total leaf DNA (~ 5 µg) was digested with *Hind*III and fragments were separated by gel electrophoresis and transferred to Hybond N (Amersham) membranes. Membranes were probed with fragments encoding portions of the *OxO, nptII* or Hyg^R coding regions. Probes were digoxigenin-labelled in PCR reactions using non alkali labile dig-dUTP and oligonucleotide primers. Probe preparation, quantification, hybridizations and detection were done according to the manufacturers instructions using a Boehringer Mannheim Dig DNA Labelling and Detection Kit. For detection of a second gene, the membranes were re-probed after being stripped 2 x 20-min each in 0.1% (w:v) SDS, 0.1X SSPE (180 µM NaCl, 10 mM $NaPO_4$ pH 7.7, 1 mM EDTA) at 100°C, and 30 min in 1% SDS/0.1x SSC .

3.3.2 Characterization of oxalate oxidase (OxO):

Extracts of germinated soybean were made by homogenizing 0.25 g of fresh tissue in 0.75 mL of S16 buffer [100 mM KAc, 3 mM MgAc, 20 mM HEPES (pH 7.5), 1 mM DTT]. Centrifugation at 16,000 x *g* produced soluble (S16, supernatant fluid) and insoluble (P16, pellet) fractions. The P19 fraction of the pellet is rich in cell wall material whereas the soluble OxO fraction represents largly vacuolar contents (Lane et al. 1992). Extracts were loaded into gel slots (10 µL/slot) and the proteins were separated by electrophoresis, transferred to nitrocellulose membranes and detected with germin specific antibodies or activity assays using oxalate-dependent production of H_2O_2 (Lane et al. 1993, Sugiura et al. 1979).

3.3.3 OxO activity assays:

Qualitative Assay: For each putative transgenic a small leaf piece (approx. 0.1-0.3 cm^2) was placed in a well of a 96-multi-well dish containing assay solution modified from Thompson et al. (1995). The assay solution penetrates the cut edge of the leaf within 10-60 min and in the presence of OxO results in a purple coloured edge, according to the following chemical reaction:



The assay solution penetrates the leaf slowly, and generally, after overnight incubation the entire leaf area is coloured deep purple. As this assay is based on oxalate-dependent production of H_2O_2 it is important to include oxalate minus controls to assess the background level of H_2O_2.

Quantitative Assays:

1. A quantitative assay adapted from Zhang et al.(1996), based on oxalate-dependent production of H_2O_2 was used to assess the relative levels of OxO activity in transgenic leaf tissue. Each of three samples (leaf punches using an eppendorf cap), were taken from a single leaf, and analysed separately. Results from the three samples of were averaged to minimize grinding variation between samples. Each frozen leaf sample was ground in liquid nitrogen, 1 mL of water was added, heated 80°C for 3 min, and centrifuged for 10 min at 16,000 x *g*. The supernatant fluid and the pellet were separated. Developing solution (40mM succinic acid /NaOH, pH 3.8, 60% (v/v) ethanol, 2 mM oxalic acid, 5 units/mL horseradish peroxidase, 20 µL/100 mL N,N-dimethylalanine, 8 mg/100 mL 4-aminoantipyrine) was added to pellets, supernate samples, blanks, and tubes containing 0.00025, 0.0005, 0.001, 0.002, 0.004, or 0.008 units of barley oxalate oxidase (Sigma O-4127) for the standard curve. Tubes were agitated at room temperature on a platform shaker for 1-2 hours depending on the level of activity observed (speed of colour development). Samples were centrifuged as above and the developer reaction was measured at A_{555} against control developer reaction.

2. A second method of quantitative analysis evaluated the release of $[^{14}C]CO_2$ from $[^{14}C]$oxalate to assess the activity of oxalate oxidase. This assay is not based on oxalate-dependent production of H_2O_2. Germinated seedlings were analysed according to Chiriboga (1966).

3.4 Efficacy of OxO in conferring white mould resistance.

3.4.1 Growth room assays:

Cotyledon and stem inoculation assays are described above in Section 2.2.

3.4.2 Confined field trials:

1998: Transgenic *Agrobacterium*-derived T3 and T4 progeny of 80-30-1 and null line 80-30-9 seed were planted in single rows with up to 60 seeds per row in white mould nurseries at Ottawa, Harrow and St. Foy and in a non-infested field at Elora. The row spacing was 50 cm at all locations except at St. Foy where it was 23 cm. Biolistic-derived progeny of 5A-1 [transgenic line (5A-1)-7, and null sib line (5A-1)-4] were planted at Ottawa.

1999: Single rows were used in Ottawa for biolistic-derived transgenics. *Agrobacterium*-derived T4 and T5 progeny of transgenic line 80-30-1, the null sib line 80-30-9, and check varieties Nattosan, Maple Arrow, OAC Shire and OAC Salem were planted in four row plots replicated 5X at Ottawa and St. Foy in white mould nurseries and at Elora in non infested fields. Furthermore, the four-row-plot design was repeated in non-infested fields at Ottawa and St. Foy to evaluate yield and other agronomic characteristics.

4. Development of molecular markers linked to genes providing resistance to *Sclerotinia sclerotiorum*

4.1 Identification of sources of white mould resistance or tolerance.

4.1.1 Field Trials:

To identify useful new sources of white mould resistance/tolerance GPCRC cooperated with public institutions across North America to screen the USDA Plant Introduction collection of Maturity Group (MG) I and II soybean lines in the field in 1996, 1997, 1998 and 1999 at Harrow. Single row two or three replicate plots spaced 60 cm were used initially with a susceptible check, Elgin87, sown every fourth row as a running control and as a source for disease spread. Three row plots with the susceptible check Elgin87 as the fourth row were used to confirm field tolerance in two or three replicate tests in 1997 - 1999. Sclerotia of the pathogen, *Sclerotinia sclerotiorum,* were spread on the soil and the soil kept moist by overhead irrigation three times weekly starting shortly before canopy closure. Disease incidence and severity were evaluated weekly. In 1999, this was supplemented by inoculating five individual plants in each row during the flowering stage by puncturing the stem with an ice-pick or other sharp object and inserting an oat kernel infected with *Sclerotinia* mycelium. The lesion length was determined ten days later. Tests grown included: in 1997 a 30-entry, 3-row, 3-rep test, a 20-entry, 3-row, 2-rep test, and a 30-entry, 1-row, 3-rep test; in 1998 a 12-entry, 3-row, 3-rep test, a 28-entry, 3-row, 2 rep test, and a 11-entry, 3-row, 2-rep test; and in 1999 a 27-entry, 1-row, 3-rep test, and a 10-entry, 3-row, 3-rep test.

4.1.2 Greenhouse screening:

The lines entered in the MG I and MG II trials in 1998 and 1999 were also screened in four replicate tests in a controlled environment growth room as described below.

4.2 Development of Recombinant Inbred Lines (RIL)

Based on the natural infection in the field and inoculation of soybean in growth room screenings, several resistant or tolerant soybean lines were identified. These were crossed with susceptible varieties and advanced breeding lines or crossed with other resistant lines. Populations were advanced without selection for white mould resistance to the F4 stage for field evaluation of white mould response. Selected F1s were backcrossed to the resistant and susceptible parent and advanced to the BC-1,F-3 stage for field evaluation of white mould response. In 1998 and 1999, 77 F4 (F5 in 1999) lines of SD970022 [Elgin87(susceptible)/PI518713(resistant)] and 74 lines of the reciprocal cross, SD970027, each in replicated single rows, were evaluated at Harrow, along with 123 lines of the BC1-F3 (F4 in 1999) lines, SD970102 and SD970103, (Elgin87/SD970022) and 76 lines of the BC1-F3 line, SD970110, (PI518713/SD970022). The F5 lines were distributed to cooperators in 1999. In addition, in 1999, the following F4 lines were field evaluated in single row plots: 38 lines of SD970129, PI189931/S19-90; 28 lines of SD970131, PI189931/Harovinton; 18 lines of PI189931/PI65454; and 19 lines of PI518713/Williams82. Field space limitations precluded growing additional material in this nursery. The PI lines and S19-90 are tolerant to white mould, while Harovinton and Williams82 are susceptible (Hoffman et al. 1999).

4.3 Molecular Marker Studies

4.3.1 Germplasm for Marker Studies:

i) White mould-tolerant lines: NKS19-90, PI 518713, PI 189931, PI 438085, PI 65454, PI 1437764 and SO8s08 which were used as parents for breeding and genetic studies

ii) White mould sensitive lines: Elgin87, Williams 82, Nattosan, Maple Glen which were used as parents for breeding and genetic studies

iii) numerous control genotypes with normal sensitivity

4.3.2. DNA Extraction:

DNA extraction was performed from frozen leaves of soybean using CTAB buffer as described by Ye (1994). Extraction buffer included 100 mM Tris-HCl (pH 8.2), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1 mM 1,10 phenanthroline, and 0.2% mercapthoethanol. Powdered leafs were incubated at 65°C for 45 min in extraction buffer. After the lysis of leaf samples, 15 ml of (25:24:1) phenol: chloroform: isoamylalcohol was mixed with the extraction buffer and centrifuged @ 10,000 x *g*. The upper phase was transferred to a new tube, and 15 ml chloroform added with mixing. After centrifugation, the upper aqueous phase was transferred to a new tube and 2.0 M NaCl was added

to the solution to dissolved polysaccharides. DNA was precipitated using one volume of isopropanol. DNA was washed twice with 75% ethanol. DNA pellets were air dried and dissolved in 500 ml of sterile water.

4.3.3 PCR Conditions:

Amplification was carried out using the method described by Cregan et al. (1998) with some modifications in reaction conditions. The thermocycler was programmed with 1 cycle of 94°C for 3 min, followed by 35 cycles of 92°C for 1min, 47°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min before holding at 4°C. Soybean SSR primers, designed by Cregan et al. (1998) and purchased from Research Genetics, Inc., were used to amplify genomic DNA. To amplify DNA, 1.5 mM $MgCl_2$, 1X buffer, 50 ng genomic DNA, 0.75 units *Taq* polymerase, and 200 mM each of dNTPs in a final volume of 20 ml were used. *Taq* polymerase, buffer, and $MgCl_2$ were purchased from Gibco-BRL. The amplified DNA was separated on a 6% polyacrylamide gel.

RESULTS AND DISCUSSION

5. Localization and quantification of oxalic acid in *Sclerotinia sclerotiorum* -infected soybeans

5.1 In vitro production of oxalic acid.

All of the *Sclerotinia sclerotiorum* isolates produced similar levels of acid during growth on agar media (data not shown). One isolate, Soy2, was therefore selected for further analysis of oxalate production *in vitro* and *in planta*. Preliminary experiments were done to assess oxalate production by isolate Soy2 during growth in minimal nutrient conditions. Within the first four days post inoculation (p.i.), the accumulation of oxalic acid in the growth medium was proportional to increases in mycelial biomass. Once sclerotial growth commenced (approx. 4 days p.i.), oxalic acid levels remained constant (Fig. 1.1). Levels of secreted oxalic acid were not significantly affected by changes in concentrations of C (glucose) or N (nitrate) sources (data not shown). These data suggested that oxalic acid accumulates extracellularly only during active mycelial growth, and are consistent with the results of a recent study in which cAMP-induced inhibition of sclerotia development was found to be associated with increased accumulation of oxalic acid (Rollins & Dickman, 1998).

5.2 Accumulation of oxalic acid in S. sclerotiorum-infected soybean tissues.

Previous studies have shown that extracellular oxalate produced by plant pathogenic fungi accumulates in diseased tissue in the form of calcium oxalate (for review see Dutton & Evans, 1996), which is insoluble in water, but soluble in dilute HCl (Budavari, 1996). Plant tissues were therefore extracted with 0.5 N HCl. The efficacy of this extraction method, and the reliability of the colorimetric-enzymatic assay for determination of oxalate levels in the acid extracts, were confirmed by HPLC identification and quantification of oxalate (data not shown).
Based on the results of our studies of *in vitro* accumulation of oxalate by isolate Soy2, and of previous studies (Maxwell & Lumsden, 1970; Magro *et al.*, 1984; Godoy *et al.*, 1990), it was anticipated that oxalate levels in infected soybean tissues would increase as fungal biomass increased. The inoculation procedure described above reliably produced disease symptoms on all tissues within 24-48 h and disease escapes were rare (data not shown). The accumulation of oxalate in *S. sclerotiorum*-infected plant tissue (growing tip, leaf, or leaf node/stem tissue) was assessed over a 96-h period, post-inoculation. At 24 h post-inoculation, oxalate levels in Soy2-infected tissue were similar to or marginally higher than levels in mock-inoculated tissue (Fig. 1.2). In all tissues tested, the concentration of oxalate increased markedly between 24 and 48 h p.i., and peaked by 72 h p.i., with levels at 96 h p.i. not significantly different from those at 72 h p.i. (Fig. 1.2 and data not shown).

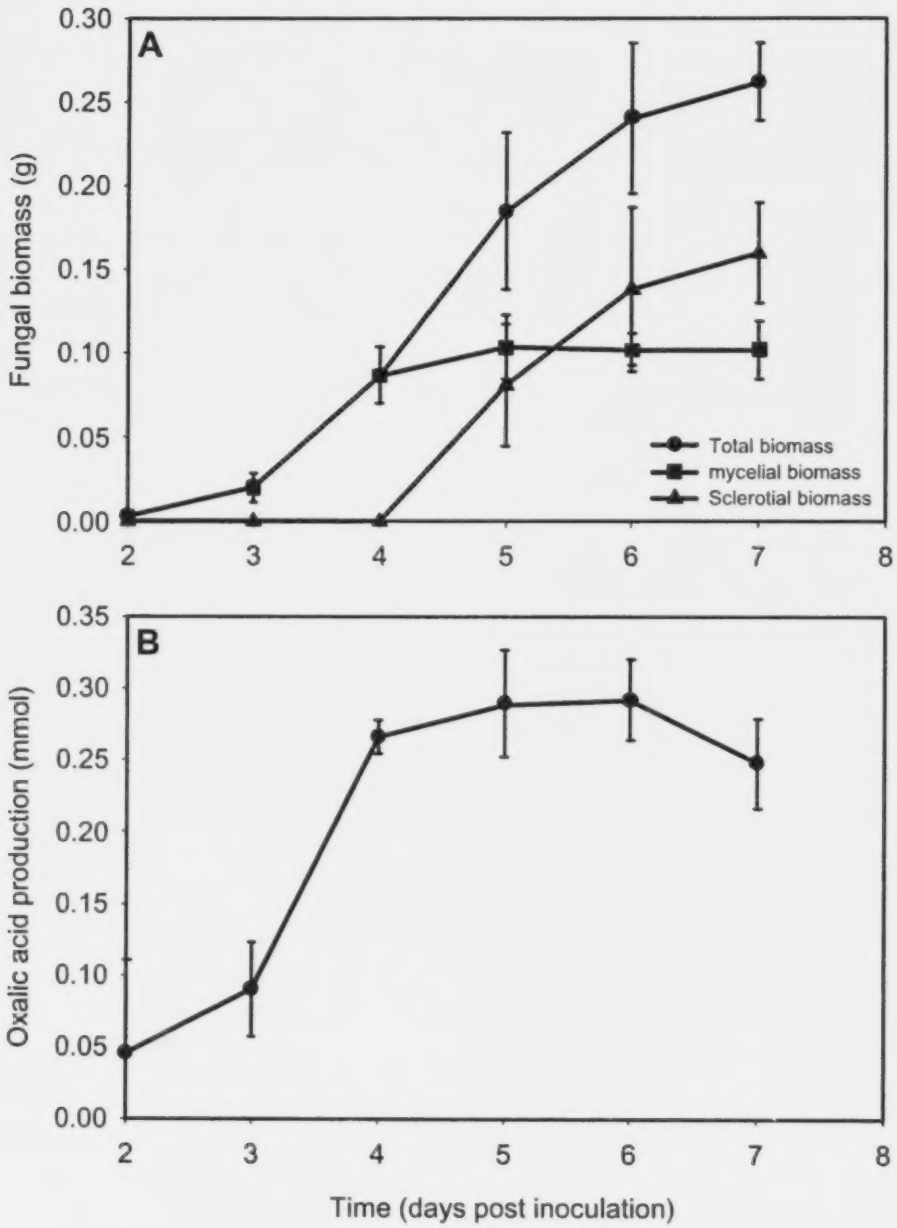


Figure 1.1 Growth and production of oxalic acid by *Sclerotinia sclerotiorum* isolate Soy2. Cultures were grown in 35 mL potato dextrose medium as described in Materials and Methods. At each time point, duplicate cultures were harvested and the fungal biomass and culture filtrate oxalate levels determined as described in Materials and Methods. A) Increase in fungal biomass over time. B) Accumulation of oxalic acid in the growth medium. The experiment was done twice. Values represent averages from four independent cultures. Bars indicate standard deviations.

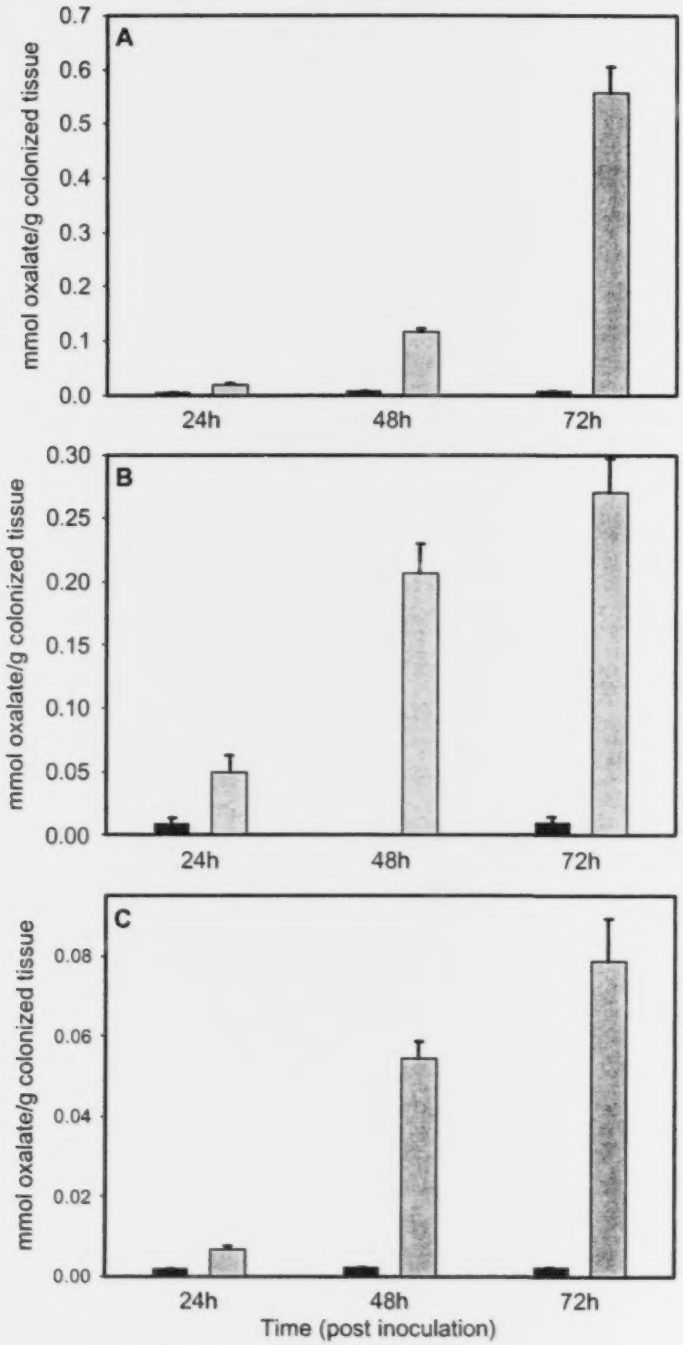


Figure 1.2. Accumulation of oxalic acid in soybean tissues infected with *S. sclerotiorum* isolate Soy2. Plants were inoculated as described in Materials and Methods. Black bars represent values for control (mock-inoculated) plants; gray bars represent values for Soy2-infected plants. Each experiment was done at least twice. Values at each time point are averages calculated from oxalate levels determined for 8 or more plants. Error bars represent SEM. A) Oxalate levels in Soy2-infected growing tips. B) Oxalate levels in Soy2-infected leaf tissue. C) Oxalate levels in Soy2-infected leaf node and adjacent stem tissue.

The oxalate levels in infected tissues were similar to those detected in culture filtrates (Fig. 1.1), but were generally higher than those reported previously for *S. sclerotiorum*-infected tissues (Maxwell & Lumsden, 1970; Magro *et al.*, 1984; Godoy *et al.*, 1990). However, it is difficult to directly compare the data obtained from all of these studies, since non-acidic extraction buffers, and a variety of different tissue homogenization procedures, were used by the different investigators.

Preliminary analysis of infected soybean blossoms suggested that the levels of oxalate accumulating in the blossoms were similar to those in growing tip (data not shown). However, only a few blossoms were available for assay. Substantially higher levels (3- to 8-fold) of oxalate accumulated in fungal-infected leaf and growing tip than in the leaf nodes and associated axil and stem tissue (Figure 1.2). It is possible that these data reflect the ability of the fungus to utilize the different tissues as a nutrient source. However, the oxalate levels in colonized leaf node/stem tissue may be artifactually low because of the difficulty in excising only necrotic tissue from this region of the plant.

6. Development of suitable screening methods for white mould resistance

6.1 Evaluation of commercial varieties and plant introductions for resistance in the field.

Evaluation of commercial varieties in the field. In 1997, plant introductions were screened in two trials consisting of maturity group I and II. Percentage infection in the Group I trial (30 entries) averaged 18.9% (range 58.7 to 0%) Elgin 87 (control, susceptible) averaged 45%. S19-90 averaged 10%. Eleven entries had fewer plants infected than S19-90 (Table 2.1) but were not significantly different. Group II trial (20 entries) averaged 19.6% infected plants (range 44% to 0%). Williams 82 (susceptible) averaged 43% infection, S19-90 averaged 11.5%. Only 6 entries had fewer plants infected than S19-90 but were not significantly different. Infection in commercial varieties was insufficient to differentiate among entries (no data presented).

Natural infection in the nursery failed to develop in 1998 and 1999. In 1999, results of inserting infected oat kernels in soybean stems resulted in inconsistent disease development. Following cut stem inoculation with agar/starch inoculum all entries were susceptible to infection and showed little resistance to disease spread. In the recommended varietie trial the remaining healthy stem tissue ranged from 19.4 cm to 0 cm in 93 entries. S19-90 (resistance) had healthy tissue measuring 8.3 cm. The average healthy stem length for the trial was 4.5 cm. Experimental lines submitted from private and public breeders (70) averaged 7.2 cm (range 18.1-0 cm). In this trial S19-90 averaged 10 cm (results not shown because of confidentiality).

6.2 Growth room evaluation of cotyledons and seedlings as indicators of resistance.

Cotyledons of selected plant introductions inoculated in the growth room were rated for disease severity at 6, 7 or 8 days following inoculation. The number of dead plants increased with time after inoculation but there was little change after 7 days (Table 2.2). PI 358318A and S19-90 were the most resistant entries in the trial. All trials involving 2-week-old seedlings and cotyledons were subsequently rated 7 days after inoculation.

The majority of plant introductions screened under field conditions was not resistant when screened in the growth room. The relative response of S19-90 (R) and Elgin 87 (S) was similar in both field and growth room. A few plant introductions were resistant in both field and growth room e.g. PI 427107A, PI 358318A, PI 437129A, PI 437451 and PI 360835 (compare Tables 2.1 and 2.3).

Removal of the growing point and primary leaves at the time of inoculation resulted in a variable response. In 2 out of 3 trials with 4 lines, removal of the growing point reduced seedlings killed (Table 2.4).

6.3 Screening transgenic material for resistance.

Several trials were conducted with ECORC transgenic material in which cotyledons and stems of seedling and 2 week old plants were inoculated in the growth room. The T_3 progeny of 80-30-1 has consistently been more resistant than the original parent (X2653-4-3-S2-2) and the T_3 null line (segregated sister line 80-30-9 with no transgene). Cotyledons of 80-30-1 senesced prior to the fungus reaching the stem, similar to the response of the commercial resistant variety S19-90. In 4 trials, the transformed lines with the "B" insertion had significantly less plant death following cotyledon inoculation than the null sister line (Table 2.5). Following inoculation of stems cut below the primary or first trifoliolate leaf, lesion development on 80-30-1 was slower than the parent line and S19-90 (Table 2.6). In a similar experiment with plants produced in growth cells, lesion development and % plants killed was less in 80-30-1 than the parental line X2653-4-3-S2-2 (Table 2.7).

Screening transgenic material for resistance in the field gave similar results to laboratory screening. The lesion length on the transgenic line 80-30-1 averaged 8.3 cm, and was significantly less than the sister null line 80-30-9 (15.6 cm) as determined from the second set of inoculations on cut stems (infection of cut stems or at nodes did not develop following the first set of inoculations in late July). It is likely that high temperatures prevented disease development.

Table 2.1. Two field trials showing percent of plant introductions (PIs) with natural white mould infection at Harrow, 1997.

Trial I			Trial II		
Entry		% infected	Entry		% infected
1234	PI437104	58.7	1234	PI438019B	44
5678	Williams82	53.7	5678	PI437475	43
910	Elgin87	46.7	910	Williams82	43
	PI464904	44		Elgin87	35.5
	Elgin87	43		Elgin87	28
	PI437451	36		PI088479	27
	PI438229	32		PI548391	25
	PI437129A	28.7		PI437437B	21
	PI358316	22		PI416748	20.5
	PI548371	20.3		PI068500	20
1112	PI416791	17.7	1112	Elgin87	18
1314	PI438085	17.7	1314	PI506477	13.5
1516	PI360835	17.7	1516	PI464917	12
1719	PI578363	15	1718	S19-90	11.5
0000	PI068454	15	5000	PI065346	10.5
0000	PI068687	14		PI479724B	6
0000	PI68551-3	13.3		PI092677	6
00	PI068457-1	11.3		PI091115	5
	S19-90	10.3		PI291309B	1.5
	PI54853	9.7		PI437495	0
	PI518713	9.3			
	PI427107A	8			
	PI088442	5.7			
	PI068670-1	5			
	PI189931	2.7			
2627	PI70016	2.7			
2829	PI437142	2.7			
30	PI437849	1.7			
	PI68610	1.3			
	PI358318A	0			
$\bar{x}$		18.9	$\bar{x}$		19.6
$LSD_{0.05}$		16.9	$LSD_{0.05}$		14.9
CV		65.7	CV		44.3

Table 2.2. Growth room assay showing percent of 14 d-old-soybean seedlings killed following inoculation of cotyledons with *Sclerotinia sclerotiorum*.

Entry		Days after inoculation			
		6	7	8	$\bar{x}$
1	S19-90	7	7	14	10
2	Elgin 87	29	40	45	38
3	PI358318A	0	0	6	2
4	PI68610	3	12	24	13
5	PI70016	32	76	76	61
6	PI189931	26	40	40	35
7	PI437142	16	25	25	22
8	PI437104	47	51	51	49
9	PI464904	63	84	84	77
10	PI438229	21	56	64	47
	$\bar{x}$	24	39	43	35

$LSD_{0.05}$ Entry X Interval = 21.1

Table 2.3. Growth room assays of three plant groups showing percent of plants killed after inoculation of cut stems with *Sclerotinia sclerotiorum*, Harrow 1998.

Group 1*		Group 2*		Group 3**	
Entry	Dead (%)	Entry	Dead (%)	Entry	Dead (%)
S19-90	31	S19-90	35	S19-90	8
Elgin 87	61	Elgin 87	78	Elgin 87	14
PI068454	81	358318A	20	Williams	65
PI427107A	41	68610	52	PI437129A	13
PI068687	78	70016	58	PI437451	3
PI437849	47	189931	69	PI358316	27
PI518713	56	437142	89	PI438085	31
PI68551-3	83	437104	57	PI416791	37
PI068670-1	89	464904	40	PI54853	29
PI068457-1	82	438229	69	PI578363	44
PI088442	79			PI548371	43
				PI360835	6
$\bar{x}$	66.2		57		26.8
$LSD_{0.05}$	12.8		12.6		24.2
CV (%)	28.6		32.7		75.6
Pr>F	0		0		0.002

* means of 3 trials, 4 replications.

** means of 1 trial, 4 replications.

Table 2.4. Effect of excising primary leaves and growing point on plant death (%) of soybean seedlings following cotyledon inoculation with *Sclerotinia sclerotiorum*.

Variety	Trial					
	I		II		III	
	C	E	C	E	C	E
S19-90	28[a]	15	7	24	14	30
Elgin 87	85	85	19	34	89	57
PI358518A	73	31	13	23	25	0
PI 437142	79	40	26	7	100	73
$\bar{x}$	66	43**	16	22*	57	40**

a = (%) seedlings killed following cotyledon inoculation (after wounding) of 7-9 day-old soybean seedlings.

C = control

E = primary leaves and growing point excised at time of inoculation.

* $P \leq 0.05$

** $P \leq 0.01$

Table 2.5. Response (% seedlings killed) of segregating transgenic lines (T_3), after cotyledon inoculations with *Sclerotinia sclerotiorum*.

Entry		Bands	Seedlings killed (%)
1	80-30-1	B	6
2	80-30-3	A	34
3	80-30-8	B	4
4	80-30-8	B + C	8
5	80-30-11	B	17
6	80-30-9	no bands	33

F= 4.05 , P>F = 0.0159, Mean = 16.5, C.V. = 78.47%, LSD 0.05 = 16.05

Means of 4 trials, up to 50 seedlings/trial.

Inoculated 9-day-old seedlings (humid chamber first 2 days), scored 5-6 d after inoculation.

Table 2.6. Cut stem inoculation assay[1] showing lesion length (cm) of transgenic and control soybean, 1-6 days after inoculation and percent dead plants after 6 days.

Entry							Dead (%)
	0 d	1 d	2 d	4 d	5 d	6 d	6 d
80-30-1	0	0.54	0.67	0.79	0.92	1.08	0
X2653-4-3-S2-2	0	1.13	1.94	3.5	4.25	4.88	62.5
S19-90	0	1.36	1.41	2	2.23	2.45	0
Elgin 87	0	1.25	2.33	3.54	4.96	5.96	50
Pr> F		0.48	0.28	0.25	0.16	0.16	0.061
$\bar{x}$		1.01	1.47	2.23	2.84	3.52	29.06
CV		70.15	79.88	86.77	83.92	86.58	125.4
LSD		0.92	1.52	2.52	3.09	3.76	47.22

[1] - 3 plants/pot, 4 pots/trt, inoculated stems of two-week-old plants cut below the first trifoliolate leaves.

Table 2.7. Lesion length (cm) on stems of soybean plants 6 days after inoculation[1].

Entry	Lesion (cm)	Dead (%)
80-30-1	0.79	0
X2653-4-3-S2-2	3.96	59
P> F	0	0.0001
$\bar{x}$	2.38	29.5
CV	30.2 %	49.9 %
$LSD_{0.05}$	0.381	13.95

[1] - stems cut below primary leaves and inoculated; plants grown in growth cells; 8 replications with 10 or less plants/rep

7. Transformation with the wheat germin *OxO* for resistance to white mould

7.1 Agrobacterium-mediated transformation.

7.1.1 Recovery of transgenic soybean plants:

Following co-culture of cotyledonary nodes of X2653-4-3-S2-2 with A. tumefaciens EHA105/*pRD400germin* the frequency of recovery of transgenic shoots was low using the protocol similar to that described previously (Donaldson et al. 2000). Table 3.1 shows four transgenic events recovered from two experiments. Putative transgenic plants and their progeny were screened with the OxO activity assay. All the primary transgenics, the T0 plants, showed a high level of OxO activity. However, the OxO activity of the T1 progeny was variable or weak in three transgenic events. Only one transgenic event, 80-30, transmitted the gene without losing enzyme activity.

7.1.2 Southern blot and segregation analysis of OxO transgenic 80-30:

Southern blot analysis of this primary transgenic plant revealed the presence of three OxO - hybridizing bands (A, B and C). The OxO transgene segregated in the eleven T1 progeny resulting in nine plants expressing OxO activity and two which were OxO negative, the null siblings. Southern blot analysis of these eleven T1 progeny using the OxO probe is shown in Fig. 3.1. Of the three hybridizing bands seen in the primary transgenic, the results showed progeny plant T1-3 had only the A band (~6 kb), plants T1-1, -2, -4, -7, -10, and -11 had only the B band (~4 kb), plants T1-6 and -8, had both B and C bands (~2.5 kb), and plants T1-5 and -9 had no bands. There were no T1 plants identified which carried only the C band. The faint bands of ~6 kb and larger, discernible in T1-1 and -11 are non-digested DNA (confirmed in Fig. 3.2). When the DNA of these plants was probed with *nptII*, hybridization was only observed in T1-6 and T1-8. Thus, although the primary transgenic was selected on the basis of kanamycin resistance, two of the T-DNAs probably integrated without the *nptII* transgene or subsequently became deleted for the *nptII* portion. Subsequently, in the next generation there are T1 plants in which the OxO transgene copy/copies has/have segregated away from the *nptII* transgene. Analysis of T2 plants derived from T1-6 and T1-8 in which the B and C band were seen to segregate, confirmed that the *nptII* transgene was present only in those individuals carrying the C *OxO* copy and it was absent from T1 and T2 plants which did not carry the C *OxO* copy.

Analysis of OxO activity in T2 progeny revealed that only 80-30-1 and 80-30-11 were homozygous for the B band (Table 3.2). Stability of the B band transmission to T2 and T3 progeny is shown in Fig. 3.2.

Table 3.1. Four X2653-4-3-S2-2 transgenic events from two *Agrobacterium* co-cultivation experiments showing Southern hybridization band numbers, OxO activity in T0 plants and their segregation in the T1 progeny.

Transgenic Plant	Southern analysis			
	OXO activity T_0 plants	(# hybridizing bands) T_0 plants	T_1 plants	OxO activity T_1 plants
80-30	+++	3	segr.	+ve or -ve correlated with SA
82-22-1	+++	0	2 bands (all)	variable - weak (one plant strong)
82-22-2	+++	2	nd	3/10 weak
82-37	+++	1	nd	2/21 weak

* Southern analysis of primary transgenic was not done as the plant was too small and fragile. Five normal seeds were recovered and germinated.

7.1.3 Growth room screening of transgenic 80-30 for resistance to white mould:

In assays using cotyledon inoculations, 80-30-derived T3 plants, carrying the 'B' *OxO* copy, were consistently more resistant to white mould than the T3 null line derived from 80-30-9. Cotyledons of 80-30-1 senesced before the fungus reached the stem (Table 2.5, Fig. 3.3), similar to the response of the commercial resistant variety S19-90 . With stem inoculations, invasion by the fungus, as measured by speed of invasion, lesion length and plant death, was inhibited in the transgenic lines derived from 80-30-1 as compared to the null sib 80-30-9, the parental line X2653-4-3-S2-2, the susceptible variety Elgin 87 and even the best resistant variety S19-90 (Tables 2.6 and 2.7, Fig. 3.4)

Fig. 3.1. Southern analysis showing segregation of the three *OxO* hybridizing bands (~6, ~4 and ~2.5 kb) of the primary 80-30 transgenic plant in eleven T1 progeny. Control, X2653-4-3-S2-2, and transgenic plant DNA was digested with *Hind*III and probed with *OxO*.

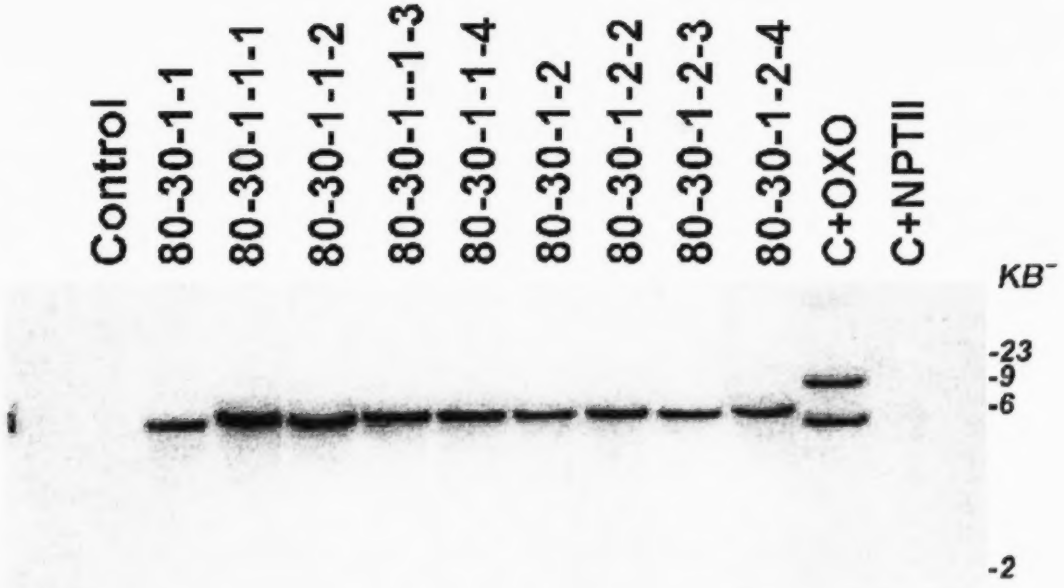


Fig. 3.2. Southern blot showing stable transmission of the ~4 kb 'B' *OxO* copy to the T2 and T3 generations of the T1 transgenic line 80-30-1. Control, X2653-4-3-S2-2, and transgenic plant DNA was digested with *Hind*III and probed with *OxO*. Lanes C+OxO and C+NPTII contain control DNA mixed with OxO or NPTII plasmids.

Table 3.2. Summary of T1 transgene segregation and T2 OxO activity of progeny from of *Agrobacterium*-derived X2653-4-3-S2-2 transformation event 80-30. Homozygous and null T1 lines are identified.

Southern results (hybridizing bands)	T_1 plants	Homozygous[a]	OxO activity in T_2 plants
None	5, 9	-	no
A band	3	[T_1-3][b]	yes
B band	1, 2, 4, 7, 10, 11	T_1-1, T_1-11, [T_1-10][b]	yes
B & C[c] band	6, 8	T_1-8 (B band)	yes

[a] Homozygosity for the OxO gene of the T_1-1 and T_1-11 was confirmed by OxO activity and the presence of the B band in Southern analysis of 11 T_2 progeny. One plant, T_1-1-1, showed an extra band, however, ten T_3 progeny showed strong OxO activity.

[b] T_1-3 and T_1-10 are most likely homozygous for OxO the gene according to the segregation data but had undergone gene silencing as indicated by OxO activity.

[c] Southern analysis of T_1-6 and T_1-8 progeny showed plants with the B & C or B bands only, but plants with C alone have not as yet been identified. In some cases the B band was altered. Four of T_1-6 and none of T_1-8 progeny lacked all three hybridizing bands.

7.1.4 Confined field testing:

Confined field trials at Ottawa, St. Foy and Elora in 1998, and at Ottawa and St Foy in 1999 showed that resistance to natural infection in white mould nurseries, as indicated by the number of plants infected and disease severity index, was significantly greater in the transgenic line 80-30-1 as compared to the null sib line 80-30-9 (Tables 3.3 and 3.4). Field inoculations at Harrow in 1998 and at Elora in 1998 and 1999 agreed with the results of the natural infections (Section 6.3 and Table 3.5 respectively). Furthermore, the inoculation assays confirmed that the susceptibility to white mould of varieties OAC Shire, Maple Arrow and OAC Salem is significantly greater than the transgenic line 80-30-1; these lines escaped natural infection in the shorter season areas (Table 3.4), perhaps due to their morphology or time of flowering.

7.1.5 Yield and agronomic characteristics of 80-30-1:

The results from the confined field trials in non-infested fields at St Foy (Table 3.6) and Ottawa (Table 3.7) showed that the yield of the transgenic line 80-30-1 and the null line 80-30-9 is essentially the same, indicating that no yield drag was imposed by the transgene. Similarly the agronomic characteristics measured at Ste Foy were essentially the same. The only significant and small differences were at Ottawa in maturity, protein and sugar.

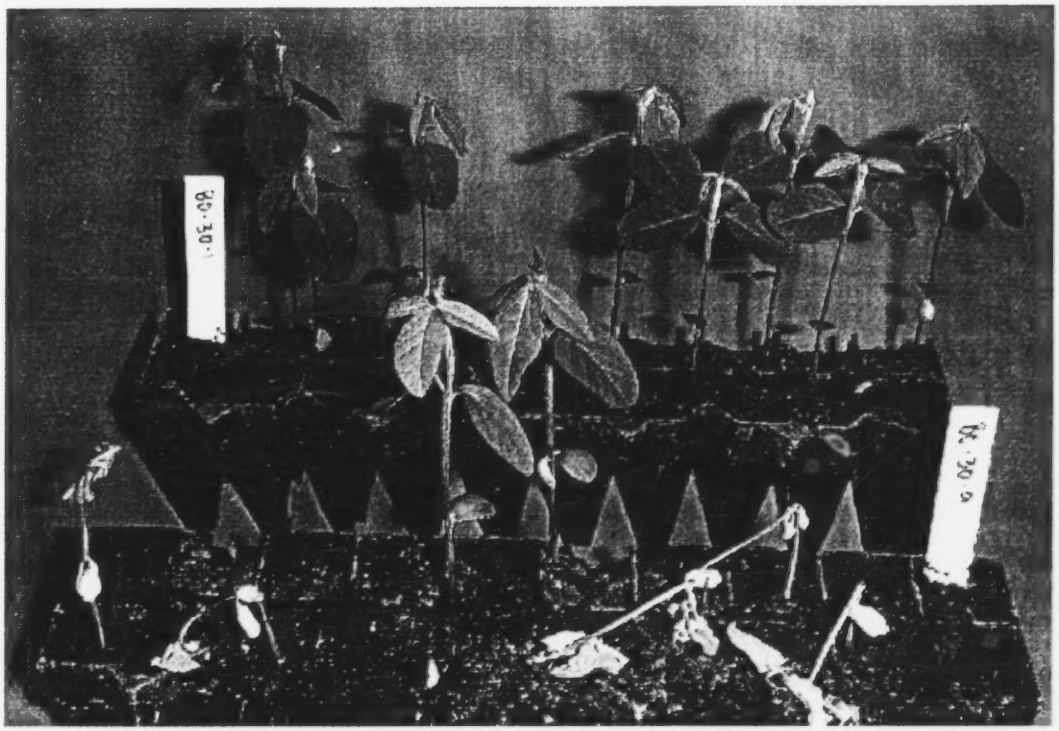


Fig. 3.3. Results of *Sclerotinia sclerotiorum* cotyledon inoculation test. Eight healthy plants in the back row are the progeny of the OxO transgenic, 80-30-1; the front row shows six dead and two yellowing control sibling 80-30-9 plants that do not carry the OxO gene.

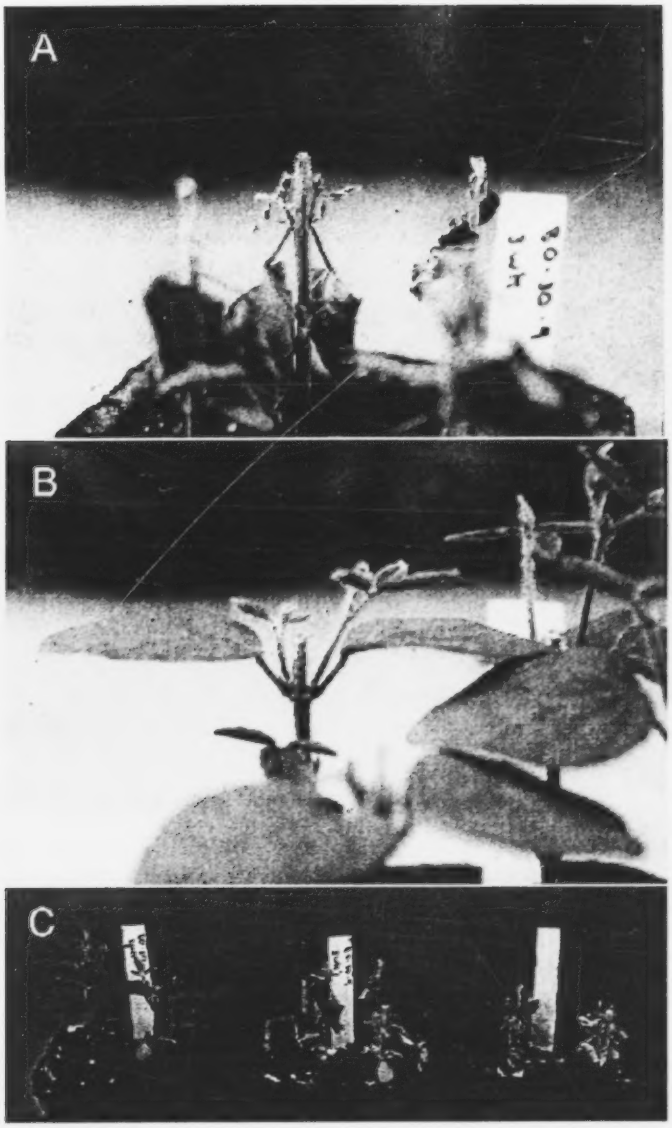


Fig. 3.4. Results of the *Sclerotinia sclerotiorum* stem inoculation assay six days after inoculation. **A**. Null sib line 80-30-9 showing long lesion. **B**. *OxO* transgenic line 80-30-1 with very short lesion. **C**. Healthy transgenic 80-30-1 derived plants and dead plants derived from 80-30-9 and the parental line X2653-4-3-S2-2 are illustrated.

Table 3.3. Disease severity index and percent plants infected in transgenic line 80-30-1 and control line 80-30-9 from trials in white mould-infested nurseries at Ottawa, Elora and St-Foy in 1998.

Genotype	Disease severity index		Plants infected	
	St-Foy	Elora	Ottawa	St-Foy
	0 to 100		%	
80-30-1	10	0	0	15
80-30-9	41	27	1	52

Table 3.4. Disease severity index, and percent plants infected for transgenic line 80-30-1 and control varieties from trials in white mould infested nurseries at Ottawa and St-Foy in 1999.

Genotype	Disease severity index			Plants infected		
	Ottawa	St-Foy	Combined	Ottawa	St-Foy	Combined
	---------- 0 to 100 ----------			---------- % ----------		
80-30-1	3	5	4	8	9	8
80-30-9	14	28	21	20	31	25
Nattosan	17	55	36	23	60	41
Maple Arrow	0	0	0	0	0	0
OAC Shire	1	24	12	2	30	16
OAC Salem	0	1	0	0	1	0
LSD 0.05	7	21	12	8	21	12

Table 3.5. Inoculation severity index from plants inoculated at Elora in 1998 and 1999.

	Inoculation severity index	
Genotype	1998	1999
	1 to 3	0 to 4
80-30-1	1.04	1.6
80-30-9	1.96	2.5
Nattosan		3.2
Maple Arrow		2.9
OAC Shire		3.9
OAC Salem		3.1
SEM	0.09	0.16

1998:
1 = 0-2 cm lesion
2 = 2-5 cm lesion
3 = 5 cm or longer lesions in most cases resulting in the death of the plant
1999:
0 = Only the wound is visible with a thin black circle around it but no tanning of the surrounding tissue.
1 = 0 - 2 cm long tanned lesion around wound
2 = 2 - 5 cm long tanned lesion around wound
3 = 5 cm or longer lesion coupled with whitening of the tissue BUT plant still alive
4 = 5 cm or longer lesion resulting in the complete death of the plant

7.2 Biolistic-mediated transformation.

The first biolistic transformation experiment, B71, using the pFF19germin plasmid, produced 19 transgenic events that showed much variation in the number of *OxO* integration sites (e.g. Fig. 3.5). Although the primary plants from all of these 19 transgenic events did show OxO activity, only 15 of the events produced fertile plants and other events lost OxO activity in T1 or the subsequent generations. A schematic representation of the Southern integration pattern of the 19 transgenic events and the OxO activity in the fertile progeny at successive generations is illustrated in Fig. 3.6. Four T0 transgenic lines were non-fertile and four did not transmit OxO activity to the T1 progeny. The T2 generations of three transgenic events had reduced fertiliy and OxO activity was weak or lost by T2 or T3 generation in three lines. Two lines [(5A-1)-7 and 5A-2] had very high OxO activity

Table 3.6. Yield and agronomic characteristics of the transgenic line 80-30-1, the null sib line 80-30-9, and check varieties grown in a non-infested field at St-Foy in 1999.

Genotype	Yield	Plant ht	Lodging	Seed		
				Weight	Protein	Oil
	kg ha^{-1}	cm	1 to 5	g 100^{-1}	%	%
80-30-1	2444	95	2.3	6.3	43.8	18.8
80-30-9	2460	97	3.0	6.6	43.8	18.7
Nattosan	2674	120	4.4	7.2	50.3	15.9
Maple Arrow	4211	107	3.6	16.0	42.0	20.7
OAC Shire	3588	119	3.6	17.6	45.6	17.9
OAC Salem	4763	104	0.5	18.2	42.9	20.6
Trans vs. null, P*	0.95	0.75	0.27	0.9	1	0.74
CV	11	5	32	5	1	2
LSD	472.5	6.9	1.1	0.7	0.6	0.4

* P level for the null hypotheses Trans = Null

Table 3.7. Yield and agronomic characteristics of the transgenic line 80-30-1, the null sib line 80-30-9, and check varieties grown in a non-infested field at Ottawa in 1999.

Genotype	Yield	Maturity	Plant ht	Lodging	Seed			
					Weight	Protein	Oil	Sugar
	kg ha^{-1}	d	cm	1 to 5	g 100^{-1}	%	%	%
80-30-1	1380	99	61	1.8	7.2	41	21.4	12.5
80-30-9	1414	101	61	2.3	7.2	41.7	21.1	12.3
Nattosan	2381	102	84	2.9	9.8	48.4	17.4	11.3
Maple Arrow	2653	100	80	1.8	18.5	40.2	23.3	12.6
OAC Shire	2957	108	79	1.5	20.4	42.2	21.5	12.2
OAC Salem	1567	99	73	1.0	17	40.4	22.8	12.8
Trans vs. null, P*	0.52	0.045	1	0.11	0.34	0.003	0.16	0.045
CV	6	1	6	23	3	1	1	1
LSD	153.6	1.8	5.2	0.5	0.5	0.3	0.3	0.2

* P level for the null hypotheses Trans = Null

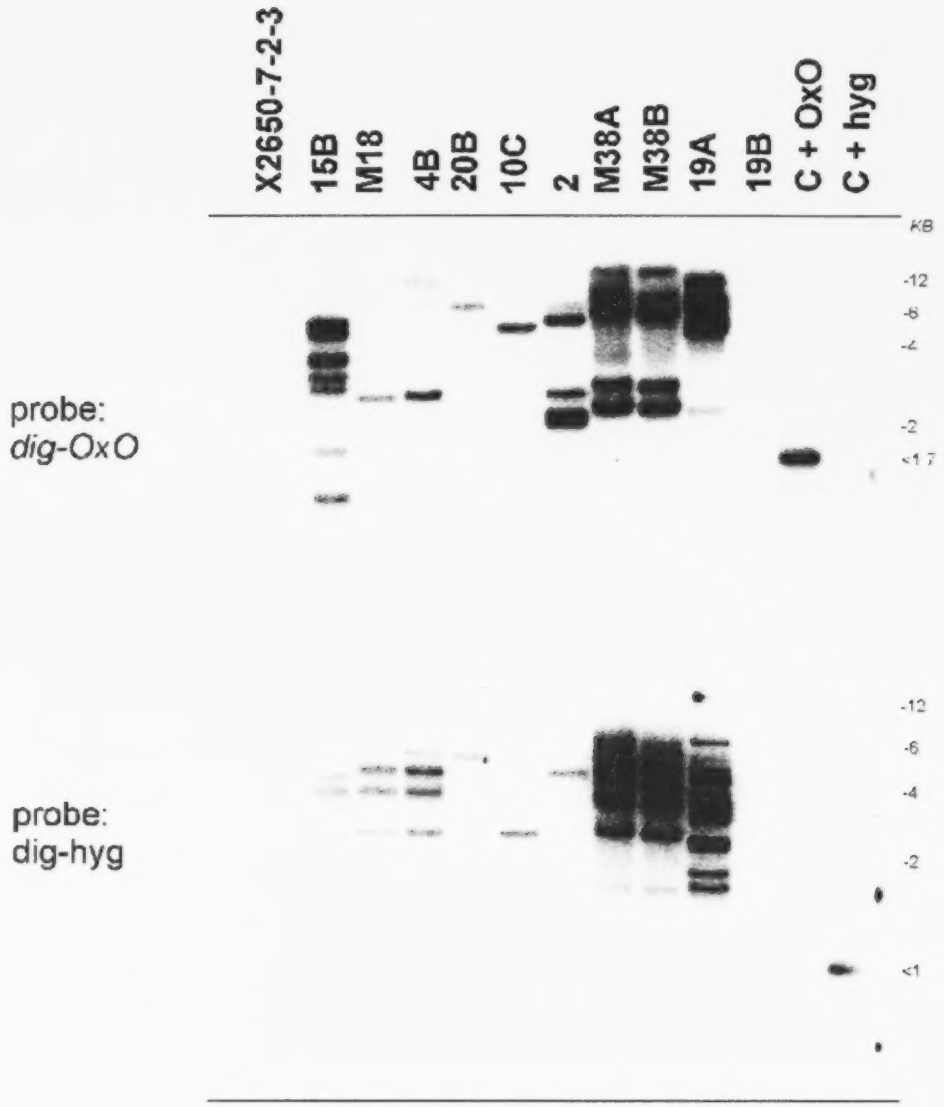


Fig. 3.5. Southern analysis of nine transgenic plants (and one escape, 19B) generated from one biolistic experiment (B71), probed with *OxO* and *Hyg*^{R}. The variation in the number of integration sites of the two transgenes is illustrated. Control, X2650-7-2-3, and transgenic plant DNA was digested with *Hind*III. Lanes C+OxO and C+hyg contain control DNA mixed with OxO or Hyg plasmids.

Fig. 3.6. Transgene activity and fertility of progeny from 19 transgenic events derived from B71 Biolistic transformation using pFF19germin. A schematic representation of the Southern banding pattern is shown below for each event.

Event	1A-1	1A-2	1B-1	1B-2	5A-1	5A-2	5A-3	5A-4	5B-1	5B-2	5B-3	5B-4	5C-1	5C-2	5D-1	5D-2	5D-3	5D-4	5D-5
# T0 plants	9	8	8	2	2	2	1	2	1	1	5	2	8	4	8	1	2	1	2
fertility	yes	low	yes	yes	yes	low	no	yes	yes	no	low	yes	yes	no	yes	yes	no	yes	low
activity in T1	weak	weak	no +DNA	no -DNA	+	+		+	+		+	+	+		no	no		+	+
activity in T2	very weak	weak			LM [1]	high / low		yes F? [3]	6		some	T1 F low	yes					yes (2)	T1 F low
activity in T3		yes/no [2]				LM			yes		no		yes[5]					yes	
activity in T4									yes[4]									yes[5]	
activity in T5									yes[4]										

[1] LM - lesion mimics, plants usually died prematurely
[2] yes / no - gene product expression segregating; line with stable enzyme activity not identified
[3] F - fertility
[4] may be showing minimal silencing
[5] two potential stable transgenic event

(see Section 7.3 and 7.4) but in the field trials they developed lesion mimics and died very soon after emergence. These two lines did not develop lesion mimics in the greenhouse where they matured normally. The high levels of OxO may have resulted in excessive production of H_2O_2 which resulted in the lesion mimic stress response in high light intensity outdoors. Good fertility and transmission of OxO activity was maintained in three transgenic events 5B-1, 5C-1 and 5D-4, that were advanced to generations T5, T3 and T4, respectively.

7.3 Characterization of the transgenic product: the 'germin' molecule.

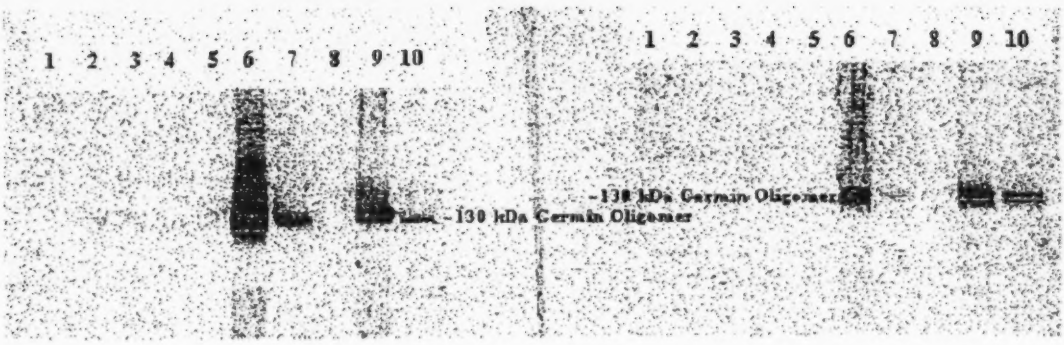
The activity of the wheat germin depends on the survival of its intact oligomeric structure that has been estimated to be a homohexamer of ~ 130 kDa comprised of monomer polypeptides (Lane et al. 1991) and N-glycan (Jaikaran et al. 1990) constituents (20 + 2 = ~22 kDa). The N-glycan moiety may serve to promote trafficking of germin through the Golgi to the cell wall (Lane et al. 1992) and may stabilize the mature protein (see Faye and Chrispeels 1989).

Wheat germin, like the germins of other domesticated cereals (barley, corn, oat, rice and rye) has unusual physicochemical properties that are not shared by other OxOs, e.g. sorghum OxO. Sorghum OxO dissociates in SDS-PAGE gels and loses activity whereas wheat germin retains its oligomeric structure and OxO activity. In addition to being resistant to SDS, the germins are extremely refractory to proteolysis by all known proteases (Grzelczak and Lane 1984), an important characteristic that is significant to their disease resistance function.

For routine characterization of germin-like OxO, SDS-PAGE is the method of choice as germin is detected on gel blots with specific antibodies or by its oxalate dependent production of H_2O_2 thus revealing its mass. The gel blots indicated that the transgenic OxO from two transgenic soybean events [80-30-1 and (5A-1)-7] is a ~130 kDa band that cross reacts with the wheat germin antiserum and is indistinguishable from wheat germin (Fig. 3.7). The gel-blot assays revealed a high level of germin OxO in the S16 fraction, however, gel-blots could not be used for quantitative germin analysis as much of the germin within the insoluble fraction, P16, is not transported into the gel (Fig. 3.7). Quantitative analysis using the '^{14}C-assay' (Table 3.8) revealed that most of the germin is localized to the insoluble fraction of cell homogenates that are rich in cell walls. Quantitative assays based on oxalate-dependent production of H_2O_2 (Section 7.4) confirmed these data except in the case of the progeny of lesion mimics 5A-1 and 5A-2.

Table 3.8. Release of [^{14}C]CO_2 from [^{14}C]oxalate in the presence of 'soluble' S16 and 'insoluble' P16 fractions of soybean homogenates of germinated seed.

Soybean Fraction	[^{14}C]CO_2 Released (cpm)	[^{14}C]oxalate Degraded (μg)
S16 - X2650-7-3-2 (parent)	21	0
S16 - X2653-4-3-S2-2 (parent)	19	0
S16 - (5A-1)-4 (OxO -)	16	0
S16 - 80-30-9 (OxO -)	15	0
S16 - (5A-1)-7 (OxO +)	2,456	2.2
S16 - 80-30-1 (OxO +)	221	0.2
P16 - (5A-1)-7 (OxO +)	21,642	19
P16 - 80-30-1 (OxO +)	7,144	6.4



A B

Fig. 3.7. A photograph of two immunoblots with a polyclonal anti-germin (anti-wheat OxO) rabbit serum.

Blot A wells were loaded with soluble 'S16' extracts of 1) X2650-7-3-2 parent control, 2) X2653-4-3-S2-2 parent control, 3) (5A-1)-4 (null), 4) 80-30-9 (null), 5) S16 buffer, 6) (5A-1)-7 (OxO transgenic), 7) 80-30-1 (OxO transgenic), 8) S16 buffer, 9) 'overproducing' tobacco OxO transgenic, 10) normal tobacco OxO transgenic.

With the exception of well # 9 all **blot B** wells were loaded with suspensions of insoluble 'P16' extracts of 1) X2650-7-3-2 parent control, 2) X2653-4-3-S2-2 parent control, 3) (5A-1)-4 (null), 4) 80-30-9 (null), 5) S16 buffer, 6) (5A-1)-7 (OxO transgenic), 7) 80-30-1 (OxO transgenic), 8) S16 buffer, 9) soluble 'S16' extract of wheat embryos, 48 h after germination, 10) suspensions of insoluble 'P16' extract of wheat embryos, 48 h after germination.

7.4 OxO activity levels of transgenic plants.

7.4.1 Agrobacterium-derived 80-30-1 progeny:

Oxalate oxidase activity levels of the 80-30-1 transgenic line plants grown in controlled environment cabinets and in the field were assessed using quantitative assays based on oxalate-dependent production of H_2O_2 in leaf discs. Comparison of OxO activity of soluble and insoluble fractions of 80-30-1 shows that most of the germin was located in the insoluble fraction of both cabinet- and field-grown plants (Table 3.9) and is in agreement with the quantitative '^{14}C-assay' (Table 3.8). Therefore only the insoluble fractions have been analysed to determine the activity range of OxO in the field grown plants. OxO activity assays from 16 plants collected on 15-7-99 show a broad range of activities, from 4.7 to 15.7 (x 10^{-3}) OxO units per leaf disc (Table 3.10). It is possible that the plants expressing OxO at the lower levels were more susceptible to white mould infection.

7.4.2 Biolistic-derived pFF19germin transformation:

(5A-1)-7 and 5A-2: These transgenic lines showing the highest OxO activity, grew well in growth cabinets and as discussed above for *(5A-1)-7*, developed lesion mimics in the field and died before the second trifoliolate leaves developed. It is interesting to note that these lines showed activity levels, in the soluble fraction and insoluble fraction that were higher by ~100-200 x and 15 x, than the same fractions of 80-30-1, respectively (Table 3.9). These were the only transgenics that exhibited a 3- to 5-fold higher activity in the soluble fraction than in the insoluble fraction. This is contrary to the soluble:insoluble ratio in the '^{14}C-assay' (Table 3.8) and may reflect differences in the developmental stage assayed.

5D-4: Similar levels of OxO activity were found in the soluble and insoluble fractions of 5D-4 progeny (Table 3.9), a ratio similar to that of wheat. It is interesting to note that the insoluble fraction activity level is similar to event (5A-1)-7, but this transgenic event did not develop lesion mimics and die, perhaps due to the relatively low soluble fraction OxO activity levels. This is a promising transgenic event because the activity appears consistent and relatively high, ranging from 19.8 to 31.7 (x 10^{-3}) OxO units per leaf disc (data from 10 field grown plants, not shown).

5B-1: OxO activity levels of 5B-1 progeny were fairly variable at the T4 generation. T5 progeny exhibited lines with low (0.5 - 3.9), mid (5.3 - 8.8) and variable (4.5 - 16.7) ranges of activity [(x 10^{-3}) OxO units per leaf disc] (data from 33 field-grown plants, not shown). The lines showing stable activity are being advanced.

5C-1: T3 progeny from six T2 lines exhibited stable and consistently high OxO activity ranging from 10.5 to 30.8 (x 10^{-3}) OxO units per leaf disc (data from 18 field grown plants, not shown). These lines are being advanced.

Table 3.9. Comparison of OxO activity levels of soluble and insoluble fractions of leaf disc homogenates from growth cabinet and field grown transgenic plants. The assay is based on oxalate-dependent production of H_2O_2.

Sample	Phenotype	total oxo units in pellet ($x10^{-3}$)	total oxo units in supernate ($x10^{-3}$)
80-30-1 cabinet A	normal	2.4 *	0.8 *
80-30-1 cabinet B	normal	1.70	0.7
20B-7 cabinet A	normal in cabinet; lesion mimic in field	39.40	181.7
20B-7 cabinet B	normal in cabinet; lesion mimic in field	38.30	199.1
80-30-1 field	normal	10.10	1.5
16(2)-3-22 field	normal in cabinet; lesion mimic in field	36.00	125.7
B71-5D-4-2-8-1 field	normal	31.70	31.8
B83-3A5-7 field	normal	31.40	23.9

* Figures shown are the means of three samples from one plant.

Table 3.10. OxO activity in the insoluble fraction of leaf disc homogenates from 16 transgenic (80-30-1) and 1 null (80-30-9) field-grown plants in 1999. Assay is based on oxalate-dependent production of H_2O_2.

Sample #	Line	OxO units (x 10^{-3})*
1	80-30-1	11.05
2	80-30-1	7.63
3	80-30-1	5.89
4	80-30-1	15.74
5	80-30-1	5.75
6	80-30-1	6.88
7	80-30-1	8.1
8	80-30-1	8.53
9	80-30-1	5.14
10	80-30-1	4.67
11	80-30-1	9.51
12	80-30-1	15.4
13	80-30-1	7.04
14	80-30-1	7.52
15	80-30-1	14.76
16	80-30-1	14.89
17	80-30-9	0

* Figures shown are the means of three samples from one plant.

7.5 Soybean transformation with pFF19soxΦAnat and pTZ19R tCUPΔOxΦAnat.

pFF19soxΦAnat: Only two transgenic lines with this plasmid were isolated as the transformation frequency had not been optimized for the host tissue (OT-95-10) at the time. However, the T1 progeny of these lines was very vigorous in 1999 confined field trials and had variable OxO activity levels. Field inoculations revealed white mould resistance in the *OxO* positive lines (data not shown). These lines are undergoing segregation and activity testing.

pTZ19R tCUPΔOxΦAnat: Twenty-two transgenic events were recovered; three were tested in field trials in 1999 and showed normal development. These lines are undergoing segregation and activity testing.

8. Development of molecular markers linked to genes providing resistance to *Sclerotinia sclerotiorum*

8.1 Field Trials.

The summers of 1998 and 1999 were very warm; the temperatures frequently rose above 30°C which exceeds the optimal temperature for *Sclerotinia sclerotiorum* development. Consequently, we were unable to obtain disease development even on the susceptible check with the distributed sclerotia and irrigation. In 1999, the stem-puncture inoculation method was successful in initiating disease, but it was too severe and all entries developed lesions of similar length.

8.2 Growth Room Tests.

Although the growth room tests gave variable results, some clear trends were discernable. Among the commercial varieties, S19-90 and Pioneer 9163 were consistently the most tolerant, whereas Elgin87 was the most susceptible. Among the PI lines, PI 507352, PI35831A and PI189931 were consistently rated as similar to or better than S19-90 and Pioneer 9163. When the tests were continued for 10 days, 57% of the PI518713 plants survived, compared with less than 15% survival for the commercial checks, including Pioneer 9163.

8.3 Development of molecular markers.

Since preparation of the proposal in early 1997, two changes have been made to the strategy used at ECORC for the "development of molecular markers linked to genes providing resistance to Sclerotinia". Originally, RFLP markers were proposed for the study since only RFLP markers had been mapped (Lorenzen et al 1995) in soybean and this would allow cross comparison to other studies. However, the publication of an enhanced USDA genetic map which integrated a large

number of SSR (Simple Sequence Repeat or Microsatellite) markers into the original RFLP (Restriction Fragment Length Polymorphism) map made it possible to shift to this more efficient class of markers. Furthermore this change allowed closer collaboration with colleagues at the GPCRC. Secondly, general mapping over the full genome of a recombinant inbred population is a large and comprehensive approach. It requires a suitable RI or F2 population, the development of which has been delayed by the challenge of reproducibly scoring white mould pathology. Therefore, a more direct strategy based on pedigrees was adopted to more rapidly identify putative QTLs which can later be validated through limited mapping of specific target regions using the recombinant inbred population.

We extracted DNA from the parental lines, Elgin87 and PI518713, at GPCRC and used over 70 Simple Sequence Repeat (SSR) as molecular markers to screen parents for polymorphisms, to identify chromosomal segments where the parental lines differ. We identified 30 SSRs polymorphic between the lines. These selected molecular markers will be used to identify quantitative trait loci (QTLs) which confer white mould resistance in the segregating populations and RILs. Without any clear disease resistance data for the RILs, there was no attempt to apply these markers to the RILs.

The SSR alleles present at 97 genetic loci were determined in 30 soybean genotypes at ECORC. The 30 genotypes included: the white mould tolerant lines, NKS19-90, PI 518713, PI 189931, PI 438085, PI 65454, PI 1437764, SO8s08; the white mould sensitive lines, Elgin87, Williams 82, Nattosan, Maple Glen and numerous control genotypes with normal sensitivity. The listed tolerant and sensitive lines include those that were used as parents for developing segregating populations for the breeding and genetic studies. The SSR loci tested were distributed across the entire soybean genome, with particular attention to SSRs known to be polymorphic in US germplasm. At the 97 SSR loci, 84 loci had multiple alleles and 13 loci had a single allele when tested across this wide germplasm pool. The identification of those SSRs that are polymorphic on the parents of each population completes the first step toward mapping those polymorphic SSR markers across the entire population. A mapped population is required for QTL analysis to identify resistance loci.

In a second strategy, allele patterns and frequencies were studied at each locus to identify chromosomal regions that may affect resistance to white mould. For example, those tolerant lines that share the same mechanism of resistance might be expected to share the same allele at a locus. At four chromosomal locations, distorted allele frequencies were observed such that tolerant lines favoured one allele and sensitive lines favoured a different allele. To be cautious, there is a statistical possibility that such a correlation has occurred by chance or through a genetic

mechanism unrelated to resistance to white mould. However, these candidate regions provide a ready starting point for focused mapping and if validated, would dramatically streamline the search for resistance loci.

CONCLUSIONS

Localization and quantification of oxalic acid in *Sclerotinia sclerotiorum*-infected soybeans

1. Oxalic acid accumulated extracelluarly only during active mycelial growth.

2. In all soybean tissues tested, oxalate did not accumulate during the first 24 h post inoculation (p.i.). The levels of oxalate increased markedly between 24 and 48 h p.i. and peaked by 72 h p.i., with levels at 96 h p.i. not significantly different from those at 72 h p.i. This is very important information for future studies on the mode of action of the *OxO* gene.

3. Greater concentrations (3- to 8-fold) of oxalate accumulated in fungal-infected leaf, petal and growing tip than in the leaf nodes and associated axil and stem tissue.

Development of suitable screening methods for white mould resistance

4. Field evaluation of PI-resistance to *S. sclerotiorum* was generally unsuccessful. Low rates of natural infection in 1998 and 1999 prevented screening of commercial lines and might have interfered with evaluating plant introductions. It is not clear why natural infection did not occur. It is suspected that high temperatures suppressed development of apothecia and development of diseases. Infection and plant loss in S19-90 (R), Elgin 87(S) and Williams(S) were low, although similar to known expected results; therefore, the trials may have overestimated resistance of some lines.

5. Inoculation of seedlings in the greenhouse and growth room was suitable for distinguishing differences between S19-90 (R) and Elgin 87 (S), as well as between transgenic 80-30-1 and the null sib line 80-30-9 or the parental line X2653-4-3-S2-2. The techniques were adequate to distinguish large differences in resistance. Growth room inoculations were not suitable for identification of all forms of resistance or tolerance or escape mechanisms that soybean lines display in the field (e.g. maturity, flower morphology and senescence, pod location, branching, etc.).

6. The inoculation medium developed in this project was suitable for obtaining consistent infection under high humidity and wet conditions. It can be applied at various rates and will adhere to most plant parts. Differences in observed disease severity between field and growth room may be related to humidity and light intensity and quality. Additional research is required on these aspects of screening for resistance to white mould.

7. The research demonstrated that transgenic lines developed at ECORC were resistant to white mould in both growth room and field. Several plant introductions expressed resistance and have been incorporated into soybean breeding programmes at GPCRC and ECORC. The new screening techniques should permit effective screening of progeny and development of commercial varieties.

Transformation with the wheat germin *OxO* for resistance to white mould

8. The germin *OxO* gene was successfully transformed into three different soybean breeding lines via *Agrobacterium*- and Biolistics-mediated transformation. Expression of OxO activity was evident in all three lines.

9. Characterization of the *OxO* transgenic product, the OxO molecule, revealed that it is homohexamer of ~ 130 kDa, indistinguishable from wheat OxO.

10. The 80-30-1 homozygous progeny from the *Agrobacterium*-derived transformant 80-30 was identified at the T1 generation. It exhibited stable expression of OxO to the T8 generation.

11. The progeny of 80-30-1 were tested for resistance to white mould in two different growth room inoculation assays and in confined field trials over two summers at a total of seven locations. In all these tests, the T3, T4 and T5 generations had much higher resistance to white mould than its parental line, the null sib line and S19-90, one of the most resistant commercial varieties. Therefore it is concluded that the *OxO* gene confers white mould resistance.

12. Confined field trials also revealed that yields from the transgenic line 80-30-1 and the 80-30-9 null sibling (without the *OxO* gene) were essentially the same, indicating no transgene yield drag.

13. Several progeny from biolistic-derived transgenics also exhibited excellent white mould resistance. However, several generations were required to identify lines with stable transmission of OxO activity.

14. Transgenic lines with at least 50% OxO activity in tissue of the insoluble fraction (i.e. walls) were white mould-resistant and performed well agronomically. The lines with most of OxO activity in the soluble fraction, had lesion mimics and died soon after germination in the field, but grew normally in growth chambers and greenhouses. This indicates the importance of cell compartmentalization of gene expression.

Development of molecular markers linked to genes providing resistance to *Sclerotinia sclerotiorum*

13. Five PI accessions were identified withs levels of resistance/tolerance superior to the best commercial varietie, S19-90.

15. F_6 recombinant lines from two crosses and F_5 RILs from two backcrosses, along with F_5 RILs with various resistance sources were developed. Field evaluation in the white mould nursery, precluded obtaining meaningful results due to record heat during the summer.

16. The SSR profile of the resistance source and the susceptible parent for the GPCRC population was determined using more than 70 SSRs. Bulked segregant analysis of these populations could not be carried out without determining the most resistant and most susceptible 10% of each population.

17. SSR alleles at 97 loci were characterized across 7 tolerant lines and 4 sensitive lines were used to create white mould plant populations at ECORC, GPCRC and the University of Guelph and compared to a range of sensitive germplasm. Identification of SSRs polymorphic between specific parents will allow mapping in those populations, once they are fully developed.

18. Analysis of the SSR allele patterns identified four candidate genomic regions that can be rapidly tested for resistance loci once the populations are ready. As the SSRs used have all been mapped on the USDA public map, these SSRs and flanking markers are readily available and suitable for molecular marker-assisted breeding for this trait.

FUTURE DIRECTIONS

1. Investigate if a relationship exists between the degree of white mould resistance and the level of OxO activity (i.e. are the lines showing higher levels of activity more resistant).

2. Characterize the mode of action of OxO (e.g. is the hypersensitive response triggered, does H_2O_2 activate a cell/plant defense response, are plant defense genes induced).

3. Determine the degree of white mould resistance conferred by the *OxO* gene under the regulation of the tCUP promoter or the superpromoter.

4. Toward obtaining complete white mould resistance, two approaches are proposed:
   i. if, as anticipated, the study on the mode of action of the *OxO* gene shows that OxO induces plant resistance genes, it would be appropriate to introgress the *OxO* gene into a soybean line containing optimal resistance genes as defined by molecular markers
   ii. pyramid onto the *OxO* transgenic line other putative resistance genes (e.g. xylanases, glucanases, chitinases)

5. Development of molecular markers will be linked to white mould resistance genes. Additional flanking SSR markers will be tested to characterize the four putative chromosomal regions, and additional dispersed SSR markers will be used to search for other putative resistance QTLs. These will then be validated by full QTL analysis on the appropriate segregating recombinant inbred mapping population. This will also determine each QTL's respective contribution to resistance levels. Markers for the most promising QTL will then be tested in molecular marker-assisted breeding for this trait.

REFERENCES

Athow, K.L. 1987. Fungal diseases. *In* SOYBEANS: Improvement, Production, and Uses (J.R. Wilcox, Ed.), pp. 687-727. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, WI.

Bevan, M. 1984. Binary Agrobacterium vectors for plant transformation. Nucl. Acids Res. 12: 8711-8722.

Baker, C.J. & Orlandi, E.W. 1995. Active oxygen in plant pathogenesis. Annu. Rev. Phytopath. 33: 299-321.

Boland, G.J. & Hall, R. 1986. Growth room evaluation of soybean varieties for resistance to *Sclerotinia sclerotiorum*. Can. J. Plant Sci. 66: 559-564.

Bradley, D.J., Kjellbom, P. & Lamb, C.J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. Cell 70:21-30.

Brisson, L.F., Tenhaken, R. & Lamb, C. 1994. Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell 6:1703-1712.

Brummer, E.C., Graef, G.L., Orf, J., Wilcox, J.R.,& Shoemaker, R.C. 1997. Mapping QTL for seed protein and oil content in eight soybean populations. Crop Sci. 37:370-378.

Budavari, S. (ed.). 1996. The Merck Index. Merck Research Laboratories: Whitehouse Station, NJ.

Chiriboga, J. 1966. Purification and properties of oxalic acid oxidase. Arch. Biochem. Biophys. 116: 516-523.

Chun, D., & Lockwood, J. L. 1984. A laboratory method of possible use in assessing resistance of soybeans to white mold. (Abstr.) Phytopathology 74: 869.

Cline, M.N. & Jacobsen, B. J. 1983. Methods for evaluation of soybean varieties for resistance to *Sclerotinia sclerotiorum*. Plant Dis. 67: 784-786.

Concibido, V.C., Denny, R.L., Lange, D.A., Orf, J.H. & Young, N.D. 1996. RFLP mapping and marker-assisted selection of soybean cyst nematode resistance in PI 209332. Crop Sci. 36:1643-1650.

Cregan, P.B., Bhagwat, A.A., Akkaya, M.S. & Rongwen, J. 1994. Microsatellite fingerprinting and mapping of soybean. Meth. Mol. Cell. Biol. 5:49-61.

Datla, R.S.S., Hammerlindl, J.K., Panchuk, B., Pelcher, L.E., & Keller W. 1992. Modified binary plant transformation vectors with wild-type gene encoding NPTII. Gene 211: 383-384.

Diers, B.W., Keim, P., Fehr, W.R. &. Shoemaker, R.C. 1992. RFLP analysis of soybean seed protein and oil content. Theor. Appl. Genet. 83:608-612.

Donaldson, P.A. & Simmonds, D.H. 2000. Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. Plant Cell Rep. 19:478-484

Dutton, M.V. & Evans, C.S. 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42, 881-895.

Faye, L. & Chrispeels, M.J. 1989. Apparent inhibition of β-fructosidase secretion by tunicamycin may be explained by breakdown of the ungycosylated protein during secretion. Plant Physiol. 89: 845-851.

Foster, E., Labbe, H., Ouellet, T., Fobert, P.R., James, L.E., Iyer, V.N., Miki, B.L. (1999) A tobacco cryptic constitutive promoter, tCUP, revealed by T-DNA tagging. Plant Molec. Biol. 41:45-55.

Hoffman, D.D., Nickell, A.D,. Nickell, C.D., Diers, B.W. & Hartman G.L. 1999. Inheritance of partial resistance to Sclerotinia sclerotiorum in soybean cultivars Asgrow A2506 and Novartis S19-90. Soybean Genetics Newsletter 26 (March 1999) [Online journal]. URL http://www.soygenetics.org/articles/sgn1999-009.html

Gamborg, OL (1982) Callus and cell culture. In LR Wetter, F Constabel, eds., Plant Tissue Culture Methods, National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan NRCC 19876

Godoy, G., Steadman, J.R., Dickman, M.B. &. Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37, 179-191.

Grzelczak, Z.F. & Lane B.G. 1984. Signal resistance of a soluble protein to enzyme hydrolysis. An unorthodox approach to isolation and purification of germin, a rare growth-related protein. Can. J. Biochem. Cell Biol. 62: 1351-1353.

Huang, H.C., Chang, C., & Kozub, G.C. 1998. Effect of temperature during sclerotial formation, sclerotial dryness, and relative humidity on myceliogenic germination of sclerotia of Sclerotinia sclerotiorum. Can. J. Bot. 76:494-499.

Jaikara, A.S.I., Kennedy, T.D., Dratewka-Kos, E. & Lane, B.G. 1990. Characterisation of the covalently bonded and adventitious glycans in germin. J. Biol. Chem. 265: 12503-12512.

Kanazin, V., Marek, L.F. & Shoemaker, R.C. 1996. Resistance gene analogs are conserved and clustered in soybean. Proc. Natl. Acad. Sci. USA 93: 11746-11750.

Kim, H.S., Sneller, C.H. &. Diers, B.W. 1999. Evaluation of soybean cultivars for resistance to sclerotinia stem rot in field environments. Crop Sci. 39:64-68.

Yu, Y.G., Buss, G.R. & Saghai Maroof, M.A. 1996. Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc. Natl. Acad. Sci. USA 93: 11751-11756.

Keen, N.T. 1992. The molecular biology of disease resistance. Plant Mol. Biol. 19:109-122.

Klement, Z. 1982. *In* Phytopathogenic Prokaryotes, M.S. Mount and G.H. Lacey, (Eds.) Academic Press, New York. Vol. 2, pp. 149-177.

Levine, A., Tenhaken, R., Dixon, R. & Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593.

Lamb, C. & Dixon, R.A. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol.48:251-275.

Lamb, C.J., Lawton, M.A., Dron, M. & Dixon, R.A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.

Lane, B.G., Grzelczak, Z.F., Kennedy, T.D., Kajioka, R., Orr, J., D'Agostino, S., & Jaikaran, A. 1986. Germin: compartmentation of two forms of the protein by washing growing wheat embryos. Biochem. Cell Biol. 64:1025-1037.

Lane, B.G., Bernier, F., Dratewka-Kos, E., Shafai, R., Kennedy, T.D., Pyne, C., Ronald Munro, J., Vaughan, T., Walters, D. & Altomare, F. 1991. Homologies between members of the germin gene family in hexaploid wheat and similarities between these wheat germins and certain *Physarum* spherulins. J. Biol. Chem. 266:10461-10469.

Lane, B.G., Cuming, A.C., Fregeau, J., Carpita, N.C., Hurkman, W.J., Bernier, F., Dratewka-Kos, E. & Kennedy, T.D. 1992. Germin isoforms are discrete temporal markers of wheat development. Eur. J. Biochem. 209:961-969.

Lane, B.G., Dunwell, J.M., Ray, J.A., Schmitt, M.R.& Cuming A.C. 1993. Germin, a protein marker of early plant development, is an oxalate oxidase. J. Biol. Chem. 268: 12239-12242.

Lee, S.H., Bailey, M.A. Mian, M.A.R., Carter Jr., T.E., Shipe, E.R., Ashley, D.A., Parrott,W.A., Hussey, R.S. & Boerma, H.R.. 1996. RFLP loci associated with soybean seed protein and oil content across populations and locations. Theor. Appl. Genet. 93: 649-657.

Lorenzen, L.L.,. Boutin, S., Young, N., Specht J.E. & Shoemaker, R.C. 1995. Soybean pedigree analysis using map-based molecular markers: I. Tracking RFLP markers in varieties. Crop Sci. 35: 1326-1336.

Magro, P., Marciano, P.& Lenna, P.D. 1984. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. FEMS Microbiol. Letts. 24, 9-12.

Mansur, L.M., Orf, J.H., Chase, K., Jarvik, T., Cregan, P.B. & Lark, K.G. 1996. Genetic mapping of agronomic traits using recombinant inbred lines of soybean. Crop Sci. 36:1327-1336.

Martens, J.W., Seaman, W.L. & Atkinson, T.G., Eds. (1988). Diseases of Field Crops in Canada, The Canadian Phytopathological Society.

Maxwell, D.P. & Lumsden, R.D. 1970. Oxalic acid production by Sclerotinia sclerotiorum in infected bean and in culture. *Phytopathology* 60, 1395-1398.

Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E. & Gelvin, S.B. 1995. Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. Plant J. 7: 661-676.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. & Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2: 225-238.

Rollins, J.A. & Dickman, M.D. 1998. Increase in endogenous and exogenous cyclic AMP levels inhibits sclerotial development in *Sclerotinia sclerotiorum*. Appl. Env. Microbiol. 64, 2539-2544.

Rongwen, J., Akkaya, M.S., Bhagwat, A.A., Lavi, U. & Cregan, P.B. 1995. The use of microsatellite DNA markers for soybean genotype identification. T.A.G. 90:43-48.

Rowe, D.E. 1993. Oxalic acid effects in exudates of *Sclerotinia trifolium* and *S. Sclerotium* and potential use in selection. Crop Sci. 33:1146-1149.

Ryals, J., Uknes, S. & Ward, E. 1994. Systemic acquired resistance. Plant Physiol. 104:1109-1112.

Simmonds, D.H. & Donaldson, P.A. 2000. Genotype screening for proliferative embryogenesis and biolistic transformation of short-season soybean genotypes. Plant Cell Rep. 19:485-490.

Staub, J.E., Serquen, F.C. & Gupta, M. 1996. Genetic markers, map construction, and their application in plant breeding. Hort. Sci. 31:729-740.

Sugiura, M.,. Yamamura, H., Hirano, R., Saski, M., Morikawa, M. and. Tsuboi, M. 1979. Purification and properties of oxalate oxidase from barley seedlings. Chem. Pharm. Bull. 27: 2003-2007.

Thompson, C., Dunwell, J.M., Johnstone, C.E., Lay, V., Ray, J. Schmitt, M., Watson, H. & Nisbet, G. 1995. Degradation of oxalic acid by transgenic oilseed rape plants expressing oxalate oxidase. Euphytica 85: 169-172.

Timmermans, M.C.P., Maliga, P., Vieira, J. & Messing, J. 1990. The pFF plasmids: Cassettes utilising CaMV sequences for expression of foreign genes in plants. J. Biotech 14:333-344.

Tu, J.C. 1985. Tolerance of white bean (*Phaseolus vulgaris*) to white mould (*Sclerotinia sclerotiorum*) associated with tolerance to oxalic acid. Physiol. Plant Pathol. 26: 111-117.

Ye, B. 1994. Mapping of verticillium resistance gene in potato by bulked segregant analysis. M.S. thesis, North Dakota State University, Fargo.

Zhang, Z., Yang, J., Collins, D.B. & Thordal-Christensen, H. 1996. Ethanol increases sensitivity of oxalate oxidase assays and facilitates direct activity staining in SDS gels. Plant Mol. Biol. Rep. 14: 266-272.

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GLOSSARY

AAFC	Agriculture and AgriFood Canada
BC	Back Cross
CO_2	Carbon dioxide
ECORC	Eastern Cereals and Oilseeds Research Centre, AAFC
GPCRC	Greenhouse and Processing Crops Research Centre, AAFC
H_2O_2	Hydrogen peroxide
MAS	Marker-Assisted Selection
MG	Maturity Group
NIL	Near Isogenic Line
NSERC	National Sciences and Engineering Research Council
PCR	Polymerase Chain Reaction
O_2	*Oxygen*
OxO	gene coding for oxalate oxidase
OxO	the enzyme oxalate oxidase
PAGE	PolyAcrilamide Gel Electrophoresis
PD	Potato Dextrose
PDA	Potato Dextrose Agar
PI	Plant Introduction
p.i.	post inoculation
QTL	Quantitative Trait Locus
RIL	Recombinant Inbred Lines
RFLP	Restriction Fragment Length Polymorphism
SCPFRC	Southern Crop Production and Food Research Centre
SDS	Sodium Dodecyl Sulphate
SSR	Simple Sequence Repeat (or Microsatellite) marker
USDA	United States Department of Agriculture